REMARKS

Amendments to the claims have been made to respond to the issues and concerns raised in the Office Action, to clarify aspects in the specification and claims, and to refine claim language. The amendments are believed to be consistent with the disclosure originally filed. The amendments also have been particularly presented to avoid, where applicable, any admission or estoppel, generally, negatively affecting the scope of protection provided by the disclosure and claims of the present application, and also in a manner that avoids prosecution history estoppel, limitation of the scope of equivalences, or the like. Any amendment should not be construed as an admission regarding the propriety of any objection or rejection raised in any Office Action, and the Applicant reserves the right to pursue the full scope of the unamended claims in any subsequent patent application as may be appropriate.

Claim 138 has been amended. Claims 1-137 have been cancelled. Claims 138-145 remain in the application. Each amendment is believed to have been made in accordance with Rule 121. However, should any unintended informality exist, it is requested that the undersigned be contacted by telephone so that it may be resolved as expediently as possible. It is believed the amendments fully respond to the issues raised in the Office Action. Further detail with respect to specific points raised in the Office Action is offered below.

The Office raises a new matter issue with respect to the recitation of "about the same number of sperm cells". The Applicant disagrees this recitation is new matter. The term "about" has an art recognized usage in the field of artificial insemination to describe numbers of sperm cells used in artificial insemination procedures. Examples of this usage may be seen in the Bakst publication (*see* pages 987-988), Chrenek publication (*see* Abstract), Conley publication (*see* page 402), Foote publication (*see* page 3075), Lukaszewicz publication (*see* Abstract, pages 165, 170-171), Sowter published patent application (*see* paragraph 0054), and Rath publication (*see* Abstract, page 202) attached to this response as Exhibit "A". Because the term "about" is used in this manner by

persons having ordinary skill in the art, such persons would understand the numbers of sperm disclosed in the Applicant's specification to include numbers of sperm that are "about" the same. Although the Applicant believes the publications cited here provide sufficient evidence of usage of the term "about" to overcome the Office's concern, the Applicant is in the process of obtaining an affidavit from a person having at least ordinary skill in the art and anticipates being able to supplement the discussion herein with the same. Accordingly, the Applicant respectfully requests the Office to withdraw its new matter concern on this point.

The Office raises a new matter issue with respect to the recitation of "at least". The Applicant disagrees that this recitation is new matter. However, it is believed the amendments to the claims resolve the Office's concern. Of course, the amendments are made solely to facilitate examination of the case and should not be construed as an admission by the Applicant.

The Office maintains an obviousness issue with respect to various combinations of references that include the Rens patent. In prior responses, the Applicant has demonstrated that the Rens patent provides no data disclosing achievement of the Applicant's claimed sort rates. To provide further evidence of this point, the Applicant notes that the elliptical nozzle of the Rens patent appears to have been further reviewed in the Rens 1999 publication attached to this response as Exhibit "B". At page 53, Rens 1999 clearly states that when the elliptical nozzle was employed in one experiment at a sample rate of 2000 sperm per second, a 54% orientation was achieved resulting in a sort rate of 200 sperm sorted in each direction. At pages 53-55, Rens 1999 clearly states that when the elliptical nozzle was employed in another experiment at a sample rate of 2000 sperm per second, greater than 50% orientation was achieved resulting in actual rates of sperm deflected and collected that were 200 per second and 180 per second. These actual sort rates achieved are well below the sort rates claimed by the Applicant, even notwithstanding the sample rates and orientation rates stated by Rens 1999. It is evident that none of the Rens documents discloses the sort rates achieved by the Applicant. This is evidence directly from the Rens documents themselves, and the Office has not

provided any arguments or evidences to refute the Applicant's evidentiary showing. Although the Applicant believes the Rens publication cited here and the Rens and Johnson patents cited in previous responses provide sufficient evidence of Rens' lack of disclosure of sort rates to overcome the Office's concern, the Applicant is in the process of obtaining an affidavit from a person having at least ordinary skill in the art and anticipates being able to supplement the discussion herein with the same.

Because the Rens patent does not disclose achievement of the Applicant's sort rates, it does not teach the element of the Applicant's claims for which it is cited and therefore cannot support the obviousness case the Office attempts to make. The examiner bears the initial burden of factually supporting any *prima facie* conclusion of obviousness. MPEP § 2142. The prior art reference (or references when combined) must teach or suggest all the claim limitations. *Id*; MPEP §2142.03. Here, Rens is cited by the Office as teaching the Applicant's claimed sort rates (*see e.g.* Office Action at page 13, "…high sorting rates, including 900 sorts per second, are suggested by the teachings of Rens"). However, the Office has not factually shown that Rens achieved the Applicant's claimed sort rates. Accordingly, the Office has not made a *prima facie* case of obviousness and its obviousness concern cannot stand.

The Office asserts it is incumbent on the Applicant to provide various kinds of declarations and evidences. However, the Applicant is not required to provide evidences unless and until the Office has made a *prima facie* case of obviousness. If the examiner does not produce a *prima facie* case, the applicant is under no obligation to submit evidence of nonobviousness. MPEP §2142. For example, the Office cites MPEP §716.02 as placing an evidentiary burden on the Applicant to establish unexpected results. However, MPEP §716.02 and the burden of establishing unexpected results is only required of the Applicant to rebut a case of *prima facie* obviousness successfully made (*see* MPEP §2142, "If, however, the examiner does produce a *prima facie* case, the burden of coming forward with evidence or arguments shifts to the applicant who may submit additional evidence of nonobviousness, such as comparative test data showing that the claimed invention possesses improved properties *not expected by the prior art*"

[emphasis added]). To require the Applicant to submit the kinds of evidentiary data requested by the Office misallocates the examination burden and in effect would be tantamount to requiring the Applicant to make a *prima facie* case of *nonobviousness*. However, this is not how the examination burden is allocated, it is the Office's responsibility to make a *prima facie* case of *obviousness*. The initial evaluation of *prima facie* obviousness thus relieves both the examiner and applicant from evaluating evidence beyond the prior art and the evidence in the specification as filed until the art has been shown to suggest the claimed invention. MPEP §2142. Accordingly, the Applicant respectfully requests the Office withdraw its obviousness concerns on this point.

The Office maintains an obviousness issue with respect to various combinations of references including Seidel '867. The Applicant believes Seidel '867 may be disqualified as prior art pursuant to 35 U.S.C. 103(c), 37 C.F.R. 1.104(c)(4), and MPEP § 706.02(l). Included with this response as Exhibit "C" is a statement pursuant to 37 C.F.R. § 1.104(c)(4)(iii) and MPEP § 706.02(l)(2). The specification additionally has been amended to disclose the names of the parties to the joint research agreement referred to in the statement. Accordingly, the Applicant respectfully requests the Office withdraw its obviousness concern regarding the combinations of references including Seidel '867.

The Applicant, having addressed each of the concerns raised in the Office Action, respectfully requests reconsideration and withdrawal of the rejections and objections to the application. Allowance of claims 138-145 is respectfully requested.

Dated this 30th day of July, 2007.

Respectfully submitted,
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Exhibit A

BIOLOGY OF REPRODUCTION 50, 987–992 (1994)

Fate of Fluorescent Stained Sperm following Insemination: New Light on Oviducal Sperm Transport and Storage in the Turkey

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ABSTRACT

A novel approach was used to evaluate the distribution of sperm in the oviduct of turkey hens inseminated before or after the onset of egg production. Prior to insemination, sperm were stained with the nuclear fluorescent stain *bis*benzimide. Sperm distribution in the sperm storage tubules (SST) of the uterovaginal junction and the infundibular tubular glands was determined by use of simultaneous differential interference contrast and fluorescence microscopy. In hens inseminated and examined prior to the onset of egg production, 94% of the SST contained sperm (21% were filled). In contrast, in hens inseminated initially before the onset of egg production and examined after the onset of lay, only 73% of the SST contained sperm (5% were filled); and in hens inseminated initially after the onset of lay and then examined, 78% of the SST contained sperm (4% were filled). Sperm were sparsely distributed in the infundibular tubular glands. Therefore, lower percentages of filled SST were associated with the onset of egg production, an indication that the sperm storage capacity of the SST is diminished with the onset and continuation of egg production. Physical events associated with the daily ovulatory cycle, such as rotation of the egg mass during shell formation, may both displace sperm residing in the SST and diminish the efficacy of sperm entry into the SST.

INTRODUCTION

The commercial turkey industry relies exclusively on artificial insemination (AI) for the production of fertile eggs (for review see Bakst [1]). Typically, semen is collected, diluted, and inseminated either fresh (less than 1 h after collection) or after storage (no more than 8 h). Hens are initially inseminated with about 200 million sperm about 14–16 days after the onset of photostimulation (light is boosted from 6 to 14 h) to assure maximal filling of the sperm storage tubules (SST) just prior to the onset of egg production. Once in egg production, hens are inseminated weekly in order to maintain a population of sperm within the SST. While the hen is in egg production, resident sperm are slowly but continuously released from the SST to assure an adequate population at the site of fertilization in the anterior end of the oviduct, the infundibulum. It is this capacity of the hen to store sperm in, and release sperm from, the SST which permits the fertilization of a daily succession of ova over several days to weeks in the absence of a mechanism that synchronizes ovulation with copulation [1–3].

Previous investigators estimated the sperm storage capacity of the SST by examining histological sections of the uterovaginal junction (UVJ) and categorizing the sperm distribution within individual SST cross-sections by labeling them either as full, partially full, or empty [4–6], or as with or without sperm [7] (Fig. 1). While this method provided some insight into sperm distribution within the SST, given their length (as long as 250 μm) and serpentine configuration, it was not only likely but highly probable that different cross-sections of the same SST were scored differently within single sections. Furthermore, since serial sections of the UVJ and distal infundibulum were not assessed, the examination of single sections of histological preparations failed to adequately reveal the spatial distribution of sperm within the SST. Likewise, because of the relatively low numbers of sperm residing in the turkey infundibular tubular glands, sperm were rarely observed in histological sections (Fig. 2).

The use of differential interference contrast (DIC) microscopy in the examination of unfixed, squash preparations of UVJ mucosa containing SST permits visual access to the whole SST including the lumen [3, 8]. In an examination of an individual SST through a series of focal planes (optical sectioning), single as well as clusters of sperm can be observed residing within the SST lumen. However, visualization of the SST lumen is impaired if the squash preparation is too thick or if the SST epithelial cells are heavily laden with lipid. When sperm stained with the fluorescent nuclear stain *bis*benzimide (Hoechst 33342) were inseminated, sperm residing within the oviducal sperm storage sites were relatively easy to locate in the squash preparations examined by dual DIC and fluorescence microscopy. The current study used these new techniques to reevaluate the distribution of sperm in the SST and infundibular tubular glands.

MATERIALS AND METHODS

Hen Groups, Sperm Staining, and AI

A total of 52 commercial-strain turkey breeder hens were used in this study. Hens were caged individually in a controlled-environment facility and provided feed and

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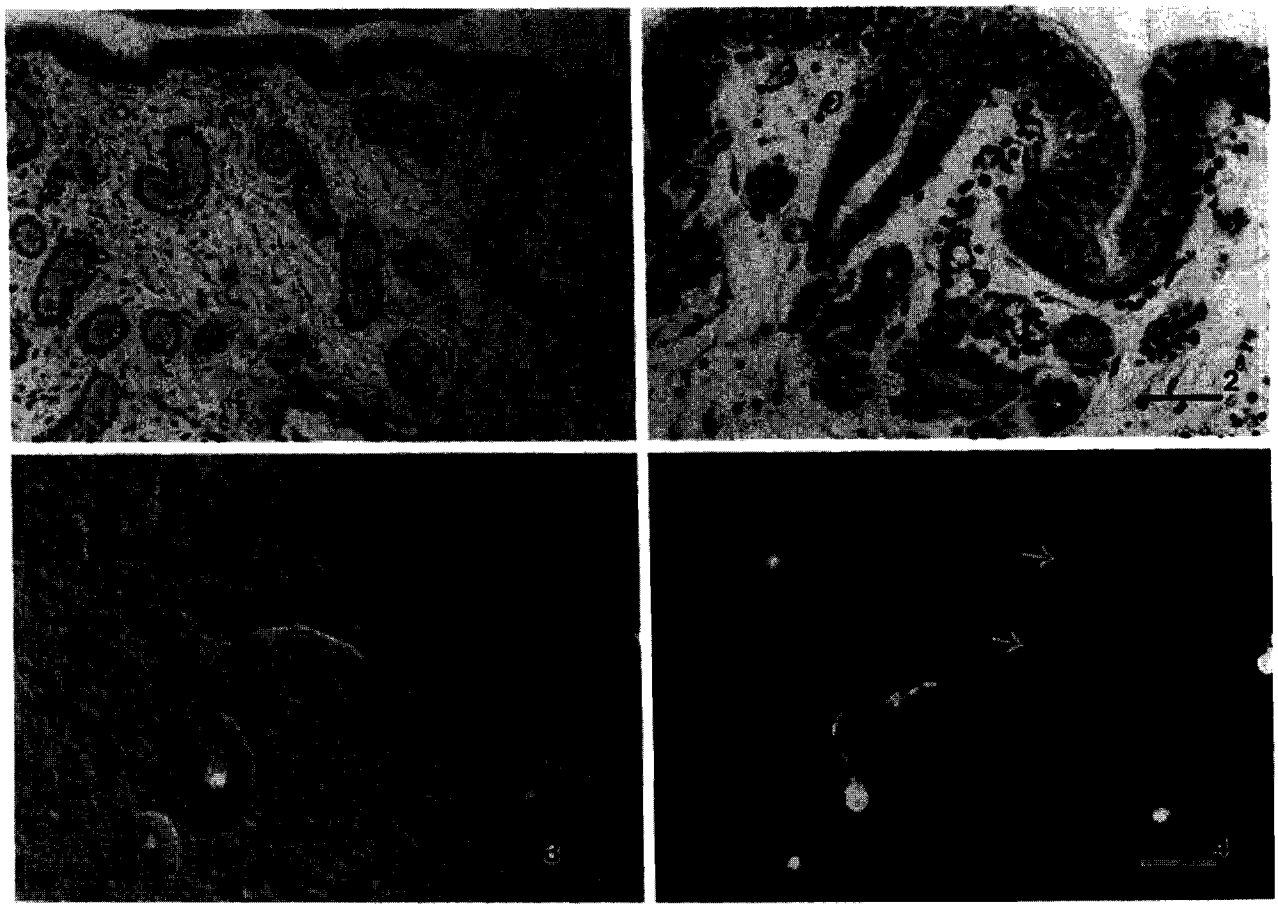

PLATE I.

FIG. 1. Histological section of UVJ mucosa revealing numerous SST. The SST have been sectioned in different planes making it difficult to ascertain both the precise number of individual SST and the distribution of sperm within the individual SST. According to the criteria presented in the *Introduction*, arrow (1) indicates a "filled" SST and arrow (2) indicates partially filled SST. Scale bar = 50 μm.

FIG. 2. Histological section of infundibular mucosa containing subepithelial tubular glands. A single sperm head (arrow) is observed within one of the tubular glands. Scale bar = 50 μm.

FIG. 3. Dual DIC and fluorescence micrograph revealing clusters of sperm at the distal ends of several SST. Not readily apparent are sperm dispersed throughout the lumina of the SST. Scale bar = 50 μm.

FIG. 4. Fluorescence micrograph of the same specimen shown in Figure 3. Note the distribution of both small clusters and single dispersed sperm throughout the lumen of the "partially" filled SST in the center of the micrograph. Scale bar = 50 μm.

water ad libitum. At 30 wk of age, the photoperiod was increased from 6 h to 14 h of light (lights-on: 0300–1700 h). Males of the same commercial strain were maintained in pens and provided feed and water ad libitum. At 26 wk of age the photoperiod was increased from 6 h to 14 h of light (lights-on: 0300–1700 h). Semen was manually collected from all males at least twice before the semen was used for AI.

For each AI, semen was manually collected from a minimum of 10 males, pooled, and diluted 1:1 with Beltsville Poultry Semen Extender (Tri Bio Laboratories, State College, PA.). The diluted semen was then diluted again with an equal volume of semen diluent containing 120 μg *bis*benzimide (Hoechst 33342; Sigma, St. Louis, MO) per ml diluent and placed on a rotary shaker (150 rpm) for 2 h at room temperature. (Preliminary trials indicated that 60 μg *bis*benzimide per ml of diluted semen incubated as described above resulted in optimal staining of the sperm with minimal effect on sperm quality.)

Pre-lay hens were inseminated initially twice before the onset of egg production, Days 14 and 15 after the onset of photostimulation, and weekly thereafter with about 100×10^6 stained sperm per insemination. Post-lay hens were inseminated twice in their first week of egg production and weekly thereafter with 100×10^6 stained sperm.

A fertility trial was conducted to determine the effect of the *bis*benzimide on sperm fecundity. Hens used in the fertility trial, which was conducted over the first 4 wk of egg production, were inseminated as described above for "pre-lay hens" with either *bis*benzimide-stained or unstained

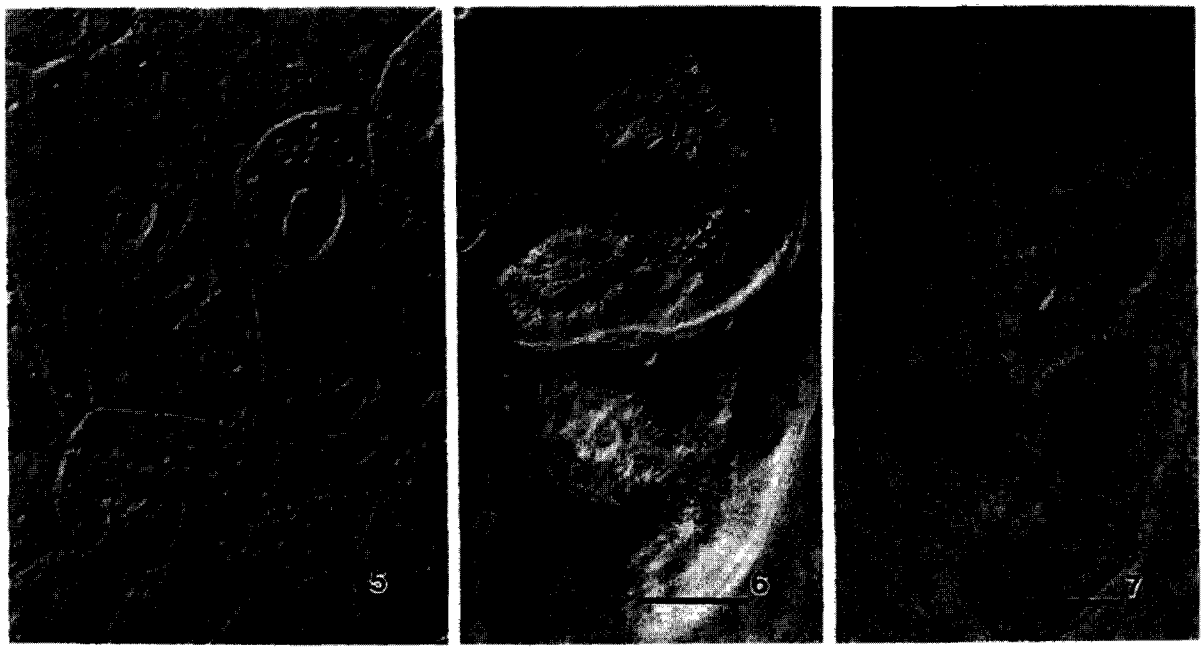

PLATE II.

FIG. 5. DIC image of the mucosa subjacent to the surface epithelium lining the infundibulum reveals relatively short infundibular tubular glands. The epithelium forming these tubular glands lacks secretory granules. Capillaries and a variety of loose connective tissue cells are also shown. Scale bar = 65 μm.

FIG. 6 Dual DIC and fluorescence micrograph of two infundibular tubular glands. Most of the epithelial cells forming these tubular glands possesses some secretory granules in their apical cytoplasm (arrow 1). Others lack secretory granules (arrow 2). Note the difficulty in discerning the fluorescent sperm in the tubular gland lumen. Compare to Figure 7. Scale bar = 30 μm.

FIG. 7. Dual DIC and fluorescence micrograph of the same specimen shown in Figure 6 except that the optics have been altered by removing the DIC analyzer. The profiles of the tubular glands are visible as is the single fluorescent sperm (arrow). Scale bar = 30 μm.

sperm. Eggs were collected daily, set in an incubator weekly, and candled for fertility after 10 days incubation.

Oviduct Preparation and Examination

Preparation of the SST for microscopy has been described in detail elsewhere [8]. At least 100 SST were evaluated in each of two mucosal folds representing the opposite sides of the UVJ. Sperm storage tubules were scored "filled" when sperm heads were densely distributed throughout the length of the SST. "Partially filled" SST possessed one or more sperm.

Preparation of the infundibular tubular glands first required the isolation of the infundibulum including the proximal 1 cm of the magnum. This was cut longitudinally, and the cut edges were pulled apart to fully expose the mucosal folds. With use of fine scissors and forceps and beginning in the anterior border of the magnum (here defined as that part of the mucosal fold clearly identified as magnal tissue), individual mucosal folds were isolated (without the underlying muscularis) and transferred to a Petri dish containing cold Hanks' Balanced Salt Solution. Folds were scanned by a stereoscope, and that part of the fold (2–4 mm long) containing single and small clusters of tubular glands was isolated and prepared for DIC in the manner identical to the UVJ folds. At least 50 microscope fields containing one or more tubular glands were evaluated in each of two folds representing the opposite sides of the infundibular lumen. Histological sections were prepared according to the methods provided elsewhere [9].

Statistical Analysis

To normalize variation in the statistical analyses, the percentages of filled, partially filled, and empty SST were subject to arc sine transformation and analyzed by the general linear model procedure of the Statistical Analysis System [10]. Means were separated by use of LS Means. Differences in mean candling fertility and hatch of fertile egg values were tested by the Student's *t*-test.

RESULTS

Bisbenzimide-Stained Sperm

*Bis*benzimide readily stained the sperm nucleus. When examined within 30 sec of mixing the *bis*benzimide with the diluted semen, about 5–10% of the sperm fluoresced intensely. Since these highly fluorescent sperm were immotile, it is assumed that their plasma and nuclear membranes were in a state of degeneration and that the sperm

TABLE 1. Comparison of fertility and hatchability between hens inseminated (AI) for the first 4 wk of egg production with *bis*benzimide-stained sperm or unstained sperm.[a]

Sperm (N = number of hens AI)	Number of eggs set	Number of fertile eggs (% fertility[b])	Number of hatched fertile eggs (% hatchability[b])
Stained (N = 14)	131	111 (79%)	90 (77%)
Unstained (N = 15)	186	168 (91%)	155 (91%)
p		<0.05	NS

[a]Hens were inseminated initially twice before the onset of egg production and weekly thereafter with 100 × 10^6 sperm. Each hen served as a repetition within each group.
[b]Percentages are means of individual hen means over the 4-wk production period.

were dead before the addition of the stain. With motile sperm, *bis*benzimide first stained the distal and then the proximal end of the nucleus. Over the 2-h incubation, staining increased in intensity and progressed toward the middle of the sperm nucleus. Visual observations showed that *bis*benzimide had no obvious effect on sperm motility. Egg fertility and hatchability (a measure of embryonic mortality) was depressed as a result of inseminating *bis*benzimide-stained sperm (Table 1).

Bisbenzimide-Stained Sperm in SST

When squash preparations of UVJ containing SST were examined by use of dual DIC and fluorescence microscopy, nearly all resident sperm fluoresced (Fig. 3). The intensity of sperm fluorescence varied with the density distribution of sperm in individual SST, the lipid composition of the SST epithelium (supranuclear lipid quenched the sperm fluorescence), the loose connective tissue cell density in the UVJ mucosa, and the thickness of the squash preparation. Optimal visualization of fluorescent sperm within the SST sometimes necessitated reducing (or eliminating) the light intensity for the DIC such that those SST without resident sperm were just discernible for scoring purposes (Fig. 4). The spatial distribution of stained sperm residing in the SST did not vary from that previously described for unstained sperm [8].

Table 2 shows that 94% (21% filled and 73% partially filled) of the SST in hens inseminated and examined before the onset of egg production (group 1) contained sperm. In contrast, only 73% (5% filled and 68% partially filled) of the SST in hens inseminated before the onset of egg production and examined after the onset of egg production (group 2) possessed sperm. The percentage of SST in hens inseminated after the onset of egg production containing sperm (group 3) was 78% (4% filled and 74% partially filled). The percentages of partially filled SST were similar among the three groups. However, the percentage of filled SST in group 1 was significantly greater than that observed in groups 2 and 3. Likewise, the percentage of empty SST in group 1 was significantly less than that observed in groups 2 and 3.

TABLE 2. Mean percentages (±SEM) of filled, partially filled, and empty SST and mean number of sperm in 100 microscope fields containing at least one infundibular tubular gland in hens inseminated initially before or after the onset of egg production.

Initial inseminations	Number of hens	% SST[a]			Infundibular tubular glands
		Filled	Partially	Empty	
Pre-lay					
Examined prior to egg production (Group 1)	7	21 ± 3[a]	73 ± 5	6 ± 4[b]	3 ± 1
Examined after onset of egg production (Group 2)	6	5 ± 3[b]	68 ± 5	27 ± 4[a]	<1 ± <1
Post-lay (Group 3)	10	4 ± 2[b]	74 ± 4	22 ± 3[a]	1 ± <1

[a]Percentages were determined by evaluating at least 100 SST on each of two separate mucosal folds of uterovaginal junction mucosa. Means (nontransformed data) with different superscripts (based on analysis of arc sine transformed data) differ significantly ($p < 0.05$).

Infundibular Tubular Glands

Tubular glands were first discernible in the infundibular mucosa about 4 cm from the transitional zone between the distal infundibulum and the proximal magnum. Here, invaginations of the surface epithelium formed discrete tubular gland-like structures (Fig. 5). These cells were nonciliated, and some possessed secretory granules in their apical cytoplasm. Progressing toward the transition zone between the infundibulum and magnum, tubular glands became more densely distributed and complex in structure. Multiple rounded and/or elongated branches formed from a single invagination (Fig. 6). There was no collecting duct. The supranuclear cytoplasm of the secretory epithelium was characterized by a dense array of secretory granules. Nuclei were visible in the basal aspect of these epithelial cells.

The abundance of secretory material in the infundibular tubular gland epithelium and the sparse distribution of sperm, which when present rarely exceeded two sperm in a single gland (see Table 2), together made it difficult to visualize resident sperm by means of dual DIC and fluorescence microscopy (Fig. 6). For better observation of sperm, squash preparations were examined with the DIC analyzer removed (Fig. 7). This permitted the image of the gland to be observed without the secretory granule component occluding its lumen.

DISCUSSION

The usefulness of direct comparisons with previous studies using a histological approach to evaluate the rate of SST filling by sperm is questionable primarily because of the different procedures used to derive the data (see *Introduction*). Furthermore, hens used in the present study, compared to the hens used in the studies cited below, were the product of several generations of successful selection for increased body weight. While the commercial turkey industry has realized and continues to realize economic benefits with heavier turkeys, increased body weight has been cited as an explanation for increasingly poor reproductive performance of breeder turkeys [11]. This may be reflected in reduced oviducal sperm storage capacity.

Notwithstanding the above, the observations presented in the present study indicate that previous investigators assessing sperm distribution in histological sections of turkey SST generally underestimated the percentage of SST containing sperm. The highest estimates of SST containing any sperm reported by Verma and Cherms [12] and Van Krey et al. [4] were 57% and 42%, respectively. Although the time of insemination relative to egg production was not provided by Van Krey et al. [4], Verma and Cherms [12] inseminated hens already in egg production. This is noteworthy considering the differences in sperm numbers in the oviduct between hens inseminated either before or after the onset of egg production seen in this study and by others [13].

McIntyre and Christensen [5] were the first to examine the possibility of differential filling rates of SST in turkey hens inseminated before or after the onset of egg production. Their analyses of histological sections revealed significantly greater percentages of SST with sperm in hens inseminated before the onset of egg production (22% filled, 50% partially filled, and 28% empty) than in those inseminated after the onset of egg production (16% filled, 39% partially filled, and 46% empty).

Observations made in the present study confirm the same trend in differential filling rates of the SST. However, in the present study, the percentages of empty SST were lower and the percentages of partially filled SST higher than those observed by McIntyre and Christensen [5]. An additional comparison of the two studies shows that while the percentages of filled SST were nearly identical in hen groups inseminated before the onset of egg production, the percentage of filled SST found by McIntyre and Christensen [5] when hens were inseminated after the onset of egg production was far greater than that observed in the present study.

The depressing effect of *bis*benzimide on hen fertility and egg hatchability was not reflected in the sperm distribution and filling rates of the SST. This is evident from the discussion in the previous paragraph. Furthermore, when visually evaluated prior to insemination, *bis*benzimide-stained sperm motility is excellent. It is possible that the stain perturbs the sperm in a subtle manner that is reflected only in the depression of hen fertility and egg hatchability. Nevertheless, the magnitude of the depression was not such that it would preclude the use of *bis*benzimide-stained sperm in further studies.

Sperm release from the SST has been proposed to be either associated with oviposition and/or ovulation [6] or to proceed as a slow but continuous process [2, 14, 15]. In the present study, the decrease in the percentage of filled SST and the increase in the percentage of empty SST observed between groups 1 and 2 indicate that the sperm storage capacity of the SST is profoundly diminished after the onset of egg production. These observations, coupled with observations regarding the presence of sperm in oviducal segments throughout the 26-h ovulatory cycle [2, 16], indicate that physiological events associated with the ovulatory cycle, particularly during the 20–22-h period during which the egg mass resides in the uterus (also referred to as the shell gland), appear to affect the release of sperm residing in the SST. The persistent distention of the uterus due to the presence of the egg mass coupled with the rotation of the uterine egg mass during its residence may contribute to the displacement of sperm located in the more superficial parts of the SST.

In addition to possessing a higher number of sperm in their SST than do hens inseminated initially after the onset of egg production [13], hens inseminated initially before the onset of egg production possess a higher percentage of filled SST. The 16% difference in the percentages of filled SST between groups 1 and 2 highlights an inability of the hen in egg production to transport and store sperm from each weekly insemination as efficiently as before the onset of egg production. This inefficiency could be due to either reduced oviducal sperm transport and/or SST storage capacity or fewer functional SST [17] or, more broadly, linked to events associated with the daily ovulatory cycle of the hen [18]. For example, the proposed mechanism of sperm displacement from the SST (described above), may keep sperm entry into the SST at lower than optimal levels.

The low numbers of sperm observed in the infundibular tubular glands in each of the three groups are difficult to explain given that relatively high numbers of sperm, an average of nearly 45 000 sperm per infundibulum, were recovered from flushings of the segment [2]. Possibly additional sperm were present in that part of the transition region at the infundibulum/magnum junction, where the tubular glands were too large and densely packed for sperm to be visualized by the DIC/fluorescence microscopy procedure. Fujii and Tamura [19] noted the presence of sperm in the tubular glands obtained from the proximal end of the chicken magnum.

In preliminary work with use of *bis*benzimide-stained sperm, two hens were inseminated immediately after oviposition. Since there is an interval of about 30–60 min be-

tween oviposition and ovulation, the transport of sperm should not be influenced by the presence of an egg mass in the oviduct. When the infundibular tubular glands were examined 2 h later, the tubular glands of both hens were engorged with sperm, indicating that a rapid sperm transport mechanism exists in the turkey oviduct. The rapid transport of a vanguard population of sperm, which bypasses the UVJ and fertilizes the ovum within 15–30 min of insemination, has been reported to exist in the chicken [20]. In contrast, if hens are inseminated just before the onset of egg production, as with the group 1 hens in this study, sperm do not accrue in the infundibulum in appreciable numbers, in spite of the absence of an egg mass in the oviduct.

The presence of bundles of sperm in the infundibular tubular glands after the hens were inseminated immediately after oviposition also explains the observed clusters of several dozen sperm occasionally observed on the perivitelline layer of laid eggs (Brillard, Wishart, Bakst, personal observations). Upon subsequent ovulations, distention of the infundibular mucosa and the release of secretory material from the tubular gland epithelial cells would result in the release of bundles of sperm, which in turn would be deposited to some localized region on the perivitelline layer. If such a bundle of sperm is deposited on the perivitelline layer overlying the blastodisc, an excessive number of sperm will enter the blastodisc, thereby contributing to the possibility of early embryonic mortality. High embryonic mortality, possibly stemming from pathological polyspermy, is also a consequence of intramagnal insemination, a surgical procedure in which sperm are deposited directly into the magnum. Sperm subsequently engorge the infundibular glands in the manner described above [21].

While of limited usefulness for the poultry breeder interested in obtaining the maximum number of live poults or chicks, knowledge of this "window" in the ovulatory cycle, that period when the oviduct is empty and sperm may bypass the UVJ, has potential application to those investigating the use of sperm as vectors of foreign DNA for introduction into the ovum. Inseminating immediately after oviposition will result in a large population of fresh sperm that bypass the SST and reach the site of fertilization minutes before ovulation. The plasmalemma of sperm residing in the SST may be subject to perturbations that could affect the adsorbed DNA.

To conclude, the new approaches used to evaluate the distribution of sperm in the oviducal sperm storage sites revealed that previous investigators had underestimated the sperm content of these areas. Furthermore, although *bis*-benzimide-stained sperm have a depressing effect on hen fertility, the loss is not of the magnitude to preclude using such stained sperm to investigate events associated with oviducal sperm storage and the fertilization process.

ACKNOWLEDGMENTS

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Intensification by Intrauterine Devices of Sperm Loss from the Sheep Uterus[1]

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A plastic spiral (IUD) was inserted by surgery into one uterine horn of 105 parous ewes; 22 other ewes were sham-operated on. About 8 weeks later, each uterine horn was ligated, and 0.07 ml of fresh ram semen was deposited in the lumen. The horns were flushed 1 or 5 hr later, and intact sperm and tailless sperm heads were counted. Sperm cell loss was greater in sham-operated estrous and ovariectomized than in sham-operated luteal phase ewes, suggesting that progesterone decreased the rate of sperm loss. The presence of an IUD greatly intensified the loss of sperm in ewes of each endocrine state. Washing accumulated cellular debris from the lumens of IUD horns before depositing semen did not reduce the loss of sperm. Excising the uterus just before depositing semen largely prevented sperm loss. These results suggest that intensified sperm loss in IUD horns was caused by a change in uterine function induced by the presence of semen and not by factors already present in the uterine lumen.

The presence of an intrauterine divice (IUD) in the sheep uterus suppresses fertility by inhibiting sperm transport and ovum fertilization, probably due to the fact that an IUD causes uterine contractions to be reversed (Hawk, 1967; Brinsfield and Hawk, 1969).

In naturally mated ewes in which small numbers of sperm were transported into the area of the IUD, most of the sperm were broken into heads and tails (Hawk, 1967). When semen was deposited directly into uterine horns, much greater numbers of sperm cells disappeared or were broken within a few hours in IUD horns than in other horns. This study was done to determine whether the intensification of sperm loss by an IUD was influenced either by the endocrine state of the animal, by factors already present in the IUD horn, or by both.

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MATERIALS AND METHODS

Each IUD was made from a strip of polyethylene plastic 12 cm long, 2 mm wide, and 1 mm thick. The strips were wound spirally around glass rods and dipped in boiling water, after which the cylindrical spiral shape was maintained. Each finished IUD measured 5 cm in length and 6 to 8 mm in outside diameter, with about 5 mm between individual turns of the spiral.

An IUD was inserted into one uterine horn of each of 105 ewes, under aseptic conditions. Using sodium pentobarbital anesthesia, we made a midventral laparotomy incision, punctured the uterine wall with sharp-pointed scissors, and screwed the IUD into the uterine lumen. Each IUD was anchored in place by a loop of thread passed through the uterine wall. The puncture was closed by one stitch through the myometrium. The insertions were made near the end of an estrous cycle to permit uterine fluid to drain through the cervix as the ewe entered the follicular phase of the cycle. An IUD was inserted into one uterine horn of each of 22 ewes and removed immediately; the puncture was closed by one stitch (sham operations, Tables 1 and 2). At the time of laparotomy, both ovaries were removed from 12 ewes (Table 1). Ewes were used experimentally about 2 months after these operations.

The ewes were mature and parous, of mixed breed-

ing. They were checked for estrous behavior twice daily in response to vasectomized aproned rams. Except for the ovariectomied and luteal phase ewes, all were used on the first day of an estrous period. The luteal phase ewes were used on day 10 of the estrous cycle (day 0 = first day of estrus). Each luteal phase ewe had at least one fully developed corpus luteum on the ovary opposite to the uterine horn containing the IUD. Special attention was given to the condition of corpora lutea because an IUD in the ewe often inhibits luteal development before day 10, particularly on the ovary adjacent to the horn containing the IUD (Hawk, 1968).

Three rams having high quality semen of similar character were used interchangeably as semen donors. Semen was collected in an artificial vagina, kept at room temperature, and deposited in the uterus of a test ewe within 30 min after collection.

At insemination, the ewe was anesthetized with sodium pentobarbital, and a midventral laparotomy was made. Ligatures were placed around one or both uterine horns to prevent the drainage of semen from the test site since previous results had indicated that the intensified sperm loss caused by an IUD was localized to the section of uterine horn containing the IUD (Hawk, 1967). One ligature was passed through the intercornual ligament and tied around the horn about 1 cm posterior to the caudal end of the IUD, and another was placed loosely around the horn about 1 cm anterior to the cranial end of the IUD. A puncture was made through the uterine wall anterior to the latter ligature, a blunt needle passed into the ligated compartment, the ligature tightened around the needle, and semen deposited in the uterine lumen; the ligature was tightened further as the needle was withdrawn. Where necessary, comparable ligatures were tied around uterine horns that did not contain an IUD.

In two experiments, the uterine lumen was washed before semen deposition. The IUD was removed through a puncture hole in the uterine wall, an 18-gauge needle inserted into the posterior end of the horn, and 100 ml of sterile saline solution forced through the uterine lumen and out of the hole through which the IUD had been removed. The uterine horn was distended somewhat by the saline solution; the distention allowed the fluid to circulate among the caruncles and wash out any cellular debris adhering to the luminal epithelium.

Usually, 0.07 ml of semen containing about 150 million sperm cells was placed in each uterine horn. We had previously found that very few if any sperm were recovered 5 hr after the use of 50 thousand sperm, the approximate number found in the uterus after natural mating, while the use of 100–200 million sperm permitted comparisons of sperm loss (Hawk, 1967).

The ewes were killed 1 or 5 hr after insemination. The ligated uterine segment was clamped at each end, injected with 5 ml of 0.9% NaCl solution, and manipulated thoroughly to wash sperm and leukocytes out of the folds of the endometrium. The anterior end of the horn was cut off, and the anterior ligature removed; the segment was drained of its contents and then flushed with an additional 40 ml of saline solution.

After overnight refrigeration, the flushings were thoroughly mixed, and counts of intact and tailless sperm were made in eosinophil counting chambers 0.2 mm deep using a phase contrast microscope.

The data were analyzed statistically by analyses of variance of percentages transformed to angles and by appropriate ranking tests. The standard errors of the means listed in the tables were calculated from the original percentages.

Henceforth, ewes bearing an IUD will be termed "IUD ewes," the horn containing the IUD will be the "IUD horn," and the other the "non-IUD horn."

RESULTS

Effect of an IUD and Endocrine State of Ewes on Sperm Loss. Semen was deposited in both uterine horns of each of the ewes, 16 sham-operated on, and 36 bearing IUD horns (Table 1). In the 16 sham-operated on ewes, 55% of sperm were recovered intact from the horns into which an IUD had been inserted and removed 2 months earlier and 53% from the previously unoperated horns; data from the two horns of each sham-operated ewe were therefore averaged (Table 1).

The percentage of sperm recovered intact in the sham-operated on ewes was significantly greater in the luteal phase ewes than in the estrous ($p < .01$) or the ovariectomized ewes ($p < .01$) (Table 1, groups 1, 3, and 5).

The presence of an IUD in the uterine lumen reduced the number of sperm recovered intact in the same animal ($p < .01$ between IUD and non-IUD horns within each of groups 2, 4, and 6, Table 1). The IUD also tended to reduce the numbers of sperm recovered intact from the non-IUD horns ($p < .05$ between non-IUD horns and horns of sham-operated ewes, for all ewes represented

TABLE 1

EFFECT OF ENDOCRINE STATE OF EWES AND INTRAUTERINE DEVICES (IUD) ON PERCENTAGES OF INTACT AND TAILLESS SPERM RECOVERED FROM THE UTERINE LUMEN[a]

Experimental conditions	Ewes (No.)	Sperm deposited per horn (millions)	Sperm recovered[b] Intact (%)	Tailless (%)	Total (%)	Packed cellular debris per horn (ml)
1. Estrous: sham operated	8	119 ± 8[c]				
Both horns			41 ± 12	19 ± 9	60 ± 14	0.03 ± 0.003[c]
2. Estrous: IUD ewes	20	132 ± 11				
IUD horn			13 ± 4	35 ± 8	48 ± 14	0.15 ± 0.031
Non-IUD horn			32 ± 9	24 ± 6	56 ± 8	0.02 ± 0.004
3. Luteal phase: sham operated	4	120 ± 8				
Both horns			95 ± 2	2 ± 1	97 ± 1	0.03 ± 0.003
4. Luteal phase:						
IUD ewes	8	154 ± 18				
IUD horn			23 ± 10	14 ± 3	37 ± 12	0.09 ± 0.010
Non-IUD horn			66 ± 11	14 ± 10	80 ± 8	0.03 ± 0.004
5. Ovariectomized: sham operated	4	127 ± 9				
Both horns			42 ± 13	7 ± 2	49 ± 13	0.03 ± 0.005
6. Ovariectomized:						
IUD ewes	8	154 ± 19				
IUD horn			5 ± 3	7 ± 2	12 ± 5	0.09 ± 0.021
Non-IUD horn			32 ± 16	7 ± 4	39 ± 15	0.05 ± 0.009

[a] Five hours after depositing 0.07 ml of ram semen in ligated segments of each uterine horn.
[b] Mean percentages (ISE) based on the number of sperm placed in each uterine horn.
[c] Mean ± SE.

in Table 1). However, the comparative percentages of sperm recovered from IUD and non-IUD horns and from horns of sham-operated ewes indicated that most of the increased sperm loss caused by the IUD was localized to the IUD horn itself.

The intensified sperm loss caused by the IUD was manifested in estrous and luteal phase ewes as an increase in both breakage and disappearance of sperm, but in ovariectomized ewes only by their disappearance (Table 1, groups 2, 4, and 6). The incidence of tailless heads was appreciably higher in estrous ewes than in the others.

During the counting of spermatozoa, the numbers of phagocytized sperm cells that could be recognized by protrusion of their tails from leukocytes accounted for no more than 5% of the lost sperm cells. Since the entire sperm cell can be phagocytized, and refractility of sperm heads in leukocytes is lost in a few minutes (Reid, 1965), much more phagocytosis probably occurred than was actually seen.

After samples of the uterine flushings were taken for sperm counts, the remainder of the flushings was centrifuged, and the volume of packed debris was recorded (Table 1). The debris consisted mostly of polymorphonuclear neutrophilic leukocytes. Most of the leukocytes flushed from sham-operated ewes and from non-IUD horns had undoubtedly migrated into the uterine lumen in response to the semen. The much larger numbers of leukocytes flushed from IUD horns were composed of cells that had migrated into the lumen in re-

sponce to the semen as well as the cells which are ordinarily present around an IUD (Hawk, 1967).

The flushed uterine horns of 12 estrous and 4 ovariectomized IUD ewes, which are not listed in Table 1, were frozen on Dry Ice, and tissue cross-sections were cut on a microtome-cryostat, stained, and examined microscopically. An average of 40% of the sperm deposited in IUD or non-IUD horns could not be found in the uterine flushings. However, the average number of sperm remaining in the uterus, nearly all in the necks of glands, was calculated to be less than 0.1% of the number deposited in the uterus. Thus the inability to account for large numbers of sperm in the flushings from estrous and ovariectomized ewes was not due to failure to wash the sperm from the uterus.

Sperm Loss After Washing the Uterine Lumen or Excising the Uterus. The factors which caused intensified sperm loss around an IUD might either have been present when semen was deposited in the uterus or have been activated afterwards. Two experiments were done to distinguish between these possibilities.

Semen was deposited in one uterine horn of each of 26 ewes (Table 2). Removing the IUD immediately before insemination did not increase the recovery of intact sperm (groups 2 and 3), nor did removing the IUD and washing the uterine lumen (group 4). With the latter procedure, however, relatively large numbers of tailless heads were recovered, suggesting that complete disappearance of sperm cells may have been retarded.

Uteri were excised from eight ewes at the time semen was placed in the IUD horn. The excised uteri were incubated either in the body cavity or in oxygenated Krebs–Ringer solution at 38 C. Sperm loss was decreased greatly, especially in the former group (Table 2, groups 5 and 6).

It was considered likely that the relative importance of newly activated or already present uterine factors in causing sperm loss could be determined more precisely by autopsying ewes at a shorter time interval. In a preliminary experiment, semen was placed in the IUD and non-IUD horns of 13 estrous ewes, and sperm loss was compared 1, 2, and 4 hr later. Four or five ewes were examined at each time interval. Sperm loss from the IUD

TABLE 2

EFFECT OF REMOVING THE IUD, WASHING THE UTERINE LUMEN, OR EXCISING THE UTERUS ON PERCENTAGES OF INTACT AND TAILLESS SPERM RECOVERED FROM UTERINE LUMENS OF ESTROUS EWES[a]

Ewe group and treatment of uterine horns	Ewes (No.)	Sperm deposited per horn (millions)	Sperm recovered[b] Intact (%)	Tailless (%)	Total (%)
1. Sham operated	6	129 ± 4[c]	30 ± 15	21 ± 8	51 ± 20
2. IUD ewes, IUD in place	3	133 ± 3	11 ± 5	17 ± 5	28 ± 9
3. IUD ewes, IUD removed[d]	3	125 ± 6	7 ± 6	28 ± 7	35 ± 18
4. IUD ewes, IUD removed, lumen washed[d]	6	141 ± 9	11 ± 2	45 ± 12	56 ± 12
5. IUD ewes, uterus excised, left in body cavity	4	142 ± 6	84 ± 10	8 ± 3	92 ± 9
6. IUD ewes, uterus excised, incubated *in vitro*	4	196 ± 21	60 ± 6	7 ± 1	67 ± 7

[a] Five hours after depositing 0.06 ml of ram semen into a ligated segment of one uterine horn of each ewe.
[b] Mean percentages (± SE) based on the number of sperm placed in eacb uterine horn.
[c] Mean ± SE.
[d] Immediately before insemination.

TABLE 3

EFFECT OF EXCISING THE UTERUS AND WASHING THE UTERINE LUMEN ON PERCENTAGES OF SPERM RECOVERED 1 HR AFTER DEPOSITING SEMEN IN LIGATED UTERINE HORNS OF ESTROUS IUD EWES

Condition of the uterus	Ewes (No.)	IUD horn				Non-IUD horn			
		Sperm recovered[a]			Packed cellular debris per horn (ml)	Sperm recovered[a]			Packed cellular debris per horn (ml)
		Intact (%)	Tailless (%)	Total (%)		Intact (%)	Tailless (%)	Total (%)	
1. Intact, IUD in place	5	37 ± 13[b]	14 ± 2	51 ± 15	0.13 ± .007	93 ± 4	3 ± 1	96 ± 4	0.04 ± .004
2. Intact, IUD removed, uterus washed[c]	5	30 ± 7	13 ± 5	43 ± 10	0.10 ± .020	86 ± 8	3 ± 1	89 ± 8	0.05 ± .003
3. Excised, IUD in place	5	99 ± 2	2 ± 1	101 ± 2	0.12 ± .029	98 ± 2	1 ± 0.4	99 ± 2	0.05 ± .002
4. Excised, IUD removed, uterus washed[c]	5	92 ± 5	3 ± 1	95 ± 4	0.04 ± .006	98 ± 2	1 ± 0.2	99 ± 2	0.03 ± .004

[a] Percentages based on an average number of 122 ± 5 million sperm deposited in each uterine horn.

[b] Mean ± SE.

[c] Immediately before insemination.

horns was just as great by 1 hr as by 2 or 4 hr, no more than 33% of the sperm being recovered intact at any interval. Loss of sperm from non-IUD horns was becoming apparent by 2 and 4 hr.

The experiment outlined in Table 3 was then done. Removal of the IUD and washing of the lumen of intact uteri did not reduce sperm breakage or their disappearance within 1 hr (groups 1 and 2), indicating that sperm loss could be caused by an active response to the semen and did not depend upon uterine contents which had been washed from the lumen. Sperm loss from uteri that were excised and left in the body cavity for 1 hr was virtually nil, suggesting that the material ordinarily present around an IUD had little if any ability to cause the loss of sperm cells (groups 3 and 4). The recovery of intact sperm cells was significantly lower in IUD horns left *in situ* than in excised IUD horns ($p < .01$, groups 1, 2 vs 3, 4).

DISCUSSION

The number of sperm cells recovered from individual uterine horns varied considerably within each experimental group. Variability in rates of sperm recovery from the female reproductive tract has been noted by Quinlivan and Robinson (1969) following artificial insemination of ewes and by Chang (1956) following deposition of semen in ligated uterine horns of rabbits. Chang (1956) also reported that large numbers of sperm cells disappeared or were broken within a few hours in intact uterine horns of rabbits while very few sperm were destroyed in excised horns.

Sperm loss from the uterine lumen was clearly delayed in luteal phase ewes of the present study. Thus, sheep seem to differ from cows in this regard since Mahajan and Menge (1967b) found no marked differences between estrous and luteal phase cattle at 6, 12, 18, or 24 hr after deposition of semen.

Several investigators have reported that the leukocytic response to semen deposited in the uterine lumen of small animals is suppressed or delayed during the luteal phase of the estrous cycle or by exogenous progesterone (Marcus, 1966; Howe, 1967; Mahajan and Menge, 1967a). The data in Table 1, covering only one point in time after deposition of semen, do not indicate whether leukocytic responses to the semen might have been delayed in luteal phase ewes as compared with the others.

The agents that caused intensified loss of sperm around an IUD were not identified, but circumstantial evidence suggests that leukocytes may have been responsible. Some phagocytized sperm cells were seen, and the ability of leukocytes to phagocytize sperm cells is well known (Austin, 1957; Bedford, 1965; Moyer *et al.*, 1967). Although no evidence was found that the leukocytes normally found around an IUD possess spermicidal capabilities, the IUD appeared to intensify the leukocytic responses to semen; this effect was particularly noticeable in IUD horns from which the original cellular debris had been washed (Table 3, group 2). Precise counts of phagocytized sperm will have to be made, however, before the intensified sperm loss around an IUD can be ascribed entirely to phagocytosis.

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FERTILIZING CAPACITY OF TRANSGENIC AND NON-TRANSGENIC RABBIT SPERMATOZOA AFTER HETEROSPERMIC INSEMINATION

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Abstract

The aim of this study was to evaluate fertilizing capacity of transgenic and non-transgenic rabbits from 3 rabbit breeds (NZW, Californian and Marder) after heterospermic insemination using phenotypic (white, black and white, and blue) and genotypic (transgene – hFVIII gene) markers. Semen from males was used to make pools of 3 males (one of each breed) and to perform heterospermic insemination. Insemination was carried out in hormonally treated young females with about 10 million of spermatozoa per insemination dose (1.0 ml). Although each female was inseminated with approximately the same number of spermatozoa, sperm from one transgenic male (NZW) was dominant, significant difference was observed in the offspring among the males with similar semen quality (53% vs. 30-17%, P<0.01-0.001). Based on our results we can conclude that transgenesis had no effect on fertilizing capacity of transgenic spermatozoa during heterospermic insemination.

Key words: rabbit, transgenic animals, heterospermic insemination.

Heterospermic insemination is an effective method to assess fertilizing capacity among males. Compared with homospermic artificial insemination, heterospermic insemination provides an accurate assessment of fertilization results as it eliminates a variation in the fertility among males, season, and management practice. Generally, the identification of DNA markers and their polymorphism in different species has made feasible the use of parentage test from heterospermic insemination done among males of the same breed without differences in morphological phenotypes (6). Phenotypic markers, such as pigmentation of skin or eyes could also be used to analyse fertilizing capacity of males if the original rabbit breeds were used.

The aim of our study was to evaluate the fertilizing capacity of sperm from transgenic and non-transgenic rabbits after heterospermic insemination using phenotypic and genotypic markers.

Material and Methods

Animals. Two New Zealand White transgenic (homozygote) males (NZW-TR 1 or NZW-TR 2) with the WAP-hFVIII gene were produced as described previously by Chrenek *et al.* (3). As non-transgenic males, California (CAL 1 or CAL 2) and Marder (MAR 1 or MAR 2) were used in the experiments. The males were mature and they were housed in individual cages, under a constant photoperiod of 16 h light phase. Temperature and humidity of the building were recorded continually by means of a thermograph positioned at the same level as the cages. The rabbits were fed *ad libitum* a commercial diet and water was provided *ad libitum* with nipple drinkers.

Semen collection and evaluation. Semen was collected using artificial vagina, once a week. Each sample was evaluated for volume, motility (as the percentage of straight moving spermatocytes), concentration (number of spermatozoa/ml counted using Bürker chamber), and percentage of live spermatozoa.

Spermatozoa were assessed basing on membrane integrity using double staining with SYBR-14 and propidium iodide (Molecular Probes Inc.) as described by Gardner and Johnson (2). About 200 spermatozoa per one group were counted using a Leica fluorescent microscope at 20x or 40x magnification, with the excitation filter set at 450-490 nm and 510-580 nm.

Table 1
Alleles distribution in offspring (a - white, a^n – acromelanistic, a^m – marder)

Male	Female	Offspring
NZW-TR (aa+TR)	NZW(aa)	white colour (aa) + transgene
NZW (aa)	NZW (aa)	white colour (aa)
CAL (a^na^n)	NZW(aa)	cal. colour (a^na)
MAR(a^ma^m)	NZW(aa)	mar. colour (a^ma)

Table 2
Seminal parameters of rabbit males

Male	Motility (%) (LSM±S.E.M.)	Concentration x 10^6 (LSM±S.E.M.)	Viability of sperm (LSM±S.E.M.)
NZW-TR1	72.00±1.29	718.40±22.71	70.17±0.70
NZW-TR2	69.00±1.26	748.20±23.71	68.75±0.71
NZW1	66.00±1.06	688.50±19.71	67.15±0.51
NZW2	67.00±1.11	694.20±21.71	68.55±0.61
CAL1	74.00±1.38	702.30±21.15	71.99±0.56
CAL2	71.00±1.32	698.80±23.12	69.20±0.66
MAR1	69.00±1.24	715.20±23.79	68.15±0.72
MAR2	70.00±1.26	701.50±22.11	68.05±0.69

NZW-TR – New Zealand White transgenic, NZW- New Zealand White non-transgenic, CAL-California rabbit, MAR - Marder rabbit

Heterospermic insemination. The males from each breed were selected and only ejaculate with more than 68% motility was used to adjust the final concentration to 10 million of spermatozoa per ml of each of the males.

Using the final concentrations of spermatozoa, two groups with transgenic males (group 1 - NZW-TR1 + CAL1 +MAR1 and group 2 - NZW-TR2+CAL2+MAR2) and two groups with non-transgenic males (group 1 - NZW1 + CAL1 +MAR1 and grouop 2 - NZW2+CAL2+MAR2) for heterospermic insemination were made:

The heterospermic insemination of each group was carried out with 1 ml of extended semen using a curved pipette (IMV, France). Selected females were induced to ovulate by intramuscularly injection of 80 IU of hCG (Werfachor, Austria) and were inseminated according to colour of the vulva.

Evaluation of offspring on phenotypic marker. Phenotypic marker (pigmentation of skin) by breeding NZW, CAL or MAR males with NZW females, NZW, CAL or MAR offspring was evaluated (Table 1).

Evaluation of offspring on genotypic marker (transgene integration). Total DNA was isolated from the tissue of newborn rabbit (3). Conditions of PCR analysis of the amplification hFVIII transgene were the same as reported by Paleyanda *et al.* (4), using primers hFVIII-F: 5'-GTA GAC AGC TGT CCA GAG GAA-3' and hFVIII-R: 5'-GAT CTG ATT TAG TTG GCC CAT C-3' which define a 578 bp region of human FVIII cDNA.

Statistic. To evaluate the effect of male on seminal parameters, an analysis of variance from seminal parameters (motility, concentration and viability) was used. Data of fertility rates from heterospermic insemination were analysed by Chi-square test.

Results

All seminal parameters (motility, concentration and viability) of different male spermatozoa are showed in Table 2. No differences were found between males of different breeds and between transgenic and non-transgenic males, respectively.

Phenotypic marker (pigmentation of skin) allowed us to evaluate fertilizing capacity of the rabbit males after delivery of their offsprings (Table 3). Although each female was inseminated approximately by the same concentration of heterospermic spermatozoa (10 million), we found, based on phenotypic and genotypic markers, that sperm from transgenic male NZW-TR1 was dominant (53% vs. 30-17% respectively, $P<0.01$-0.001). The second transgenic male spermatozoa

(NZW-TR2) had also the higher efficiency of fertilizing capacity, but significant difference was observed only comparing with MAR2 male (42.5% vs. 21%. P<0.001).

To evaluate fertilizing capacity of transgenic males after heterospermic insemination, beside the phenotypic marker (white colour), the hFVIII transgene, as a genotypic marker, was also detected in offspring by PCR method. All NZW offspring born after heterospermic insemination using spermatozoa of NZW-TR1 or NZW-TR2 male were positive (Fig. 1).

To investigate fertilizing capacity of non-transgenic NZW males, the experiment was repeated using the same CAL and MAR males but with non-transgenic NZW males (Table 4). Based on number of newborn offspring with different pigmentation of skin and colour, significant differences were obtained between CAL1 and NZW1 or MAR1 (20% vs. 40%, P<0.001). In the second group, lower fertilizing capacity of spermatozoa was detected in MAR 2 male (26%) comparing with NZW2 or CAL2 (37%, P<0.05).

Table 3

Offspring rate after heterospermic insemination with semen of transgenic rabbit males

Group	Number of inseminated /litter	Number of born	NZW-TR 1	NZW-TR 2	CAL 1	CAL 2	MAR 1	MAR 2
1	10/6	47	25 (53%)[a]	-	8 (17%)[b]	-	14 (30%)[c]	-
2	10/5	33	-	14 (42.5%)[d]	-	12 (36.5%)	-	7 (21%)[e]

[a]vs[b] and [a]vs[c] and [d]vs[e] significant difference (P<0.01 and P<0.001, respectively)

Table 4

Offspring rate after heterospermic insemination with semen of non-transgenic rabbit males

Group	Number of inseminated /birth	Number of born	NZW 1	NZW 2	CAL 1	CAL 2	MAR 1	MAR 2
1	5/2	20	8 (40%)[a]	-	4 (20%)[b]	-	8 (40%)[a]	-
2	5/3	27	-	10 (37%)[c]	-	10 (37%)[c]	-	7 (26%)[d]

[a]vs[b] and [c]vs[d] significant differences P<0.001 and P<0.05.

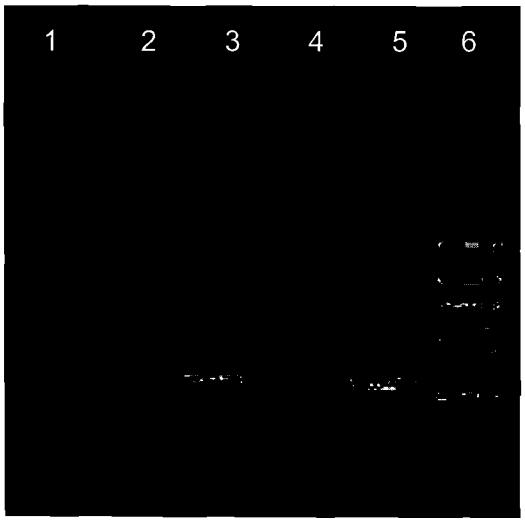

Fig. 1. Representative results of PCR analysis of hFVIII integration in offspring after heterospermic insemination. Lanes 1 and 2 – non-transgenic rabbits, lanes 3, 4 and 5 - transgenic rabbits (PCR product 578bp), lane M- Marker (GeneRuber 100bp DNA ladder plus, MBI, Fermentas, Lithuania).

Discussion

Heterospermic insemination allows evaluating differences in fertilizing capacity among males with apparently similar semen quality as for motility, concentration, and viability of spermatozoa present in insemination dose. Although each female was inseminated with approximately the same number of spermatozoa, sperm from NZW transgenic male, as demonstrated in the examination of genotypic and phenotypic markers, was dominant. Previous results reported domination of some non-transgenic rabbit male comparing with the other ones during heterospermic insemination (1, 6). Difference in fertilizing capacity in our case may be influenced by different rabbit breeds (New Zealand White, California or Marder). On the other hand, comparison of transgenic (NZW-TR1 and NZW-TR2) and non-transgenic males (NZW1 and NZW2) from NZW breed only, showed also that fertilizing capacity of spermatozoa from NZW-TR1 were dominant.

The other possibility to explain fertility differences among males may be due to maternal factors such as selective transport in the reproductive tract making differences in ovulation time inducing differences in capacitating time among sperms from different males (5). The maternal effect of NZW females used in our experiment may positively selected sperm from transgenic or non-transgenic NZW males comparing to California or Marder rabbit, however, California and Marder sperms were able to produce offspring after homospermic insemination (data not shown).

Reproductive physiological state of rabbit does at the insemination (lactating or non-lactating) and interval post-partum could affect sperm transport and change dominant relation between males (6). To eliminate this possible effect, young, but sexually adult and not yet lactating NZW females were used for heterospermic insemination after control of vaginal reaction in our experiments.

In conclusion, we showed that transgenic spermatozoa had enough fertilizing capacity during heterospermic insemination. Therefore, transgenesis had no effect on semen parameters.

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Sperm Numbers Inseminated in Dairy Cattle and Nonreturn Rates Revisited

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ABSTRACT

Three experiments were conducted to test fertility when sperm numbers per insemination ranged from 10×10^6 to 40×10^6 total sperm. All semen was from Holstein bulls that were on a regular schedule of semen collection. The semen was extended with heated homogenized whole milk, cooled, glycerolated, and frozen according to standard procedures. Semen was distributed to a large group of inseminators to minimize differential field effects on treatment. All experiments were a randomized block design, including a split plot in Experiment 2. In Experiment 1, data for 31,399 first inseminations distributed among treatments of 20×10^6, 25×10^6, 30×10^6, and 40×10^6 total sperm resulted in 69.8, 70.0, 70.1, and 70.1% nonreturns at 59 d, respectively. In Experiment 2, data for 18,197 first inseminations divided over treatments of 12×10^6, 16×10^6, and 20×10^6 total sperm resulted in 70.2, 72.4, and 70.8% nonreturns at 59 d, respectively. In Experiment 3, 38,890 first inseminations distributed over treatments of 10×10^6, 13×10^6, 16×10^6, and 20×10^6 total sperm resulted in 70.5, 72.2, 73.1, and 71.5% nonreturns at 59 d, respectively. Bull nonreturns ranged from 64 to 76% in the three trials. These results indicate that, under good conditions, total sperm numbers per straw can be reduced to 10×10^6 total sperm with a reduction of nonreturn rates at 59 d, for most bulls, of about 1 percentage unit from the maximum when professional inseminators are used.

(**Key words**: bull, sperm, insemination, nonreturn rates)

Abbreviation key: **WM** = whole milk.

INTRODUCTION

The number of sperm that are required for AI to obtain maximal fertility, considering the genetic merit of a bull and other factors, is one of the most important elements in an AI breeding program. During the development of the AI industry, a variety of factors were identified that affect level of fertility, including the inherent fertility of the bull; semen collection and processing procedures; packaging, shipping, aging of sperm before use; inseminator skill, and cow and herd management factors (17, 18). With the use of frozen semen, changes in sperm during storage were reduced. However, differential resistance of sperm from individual bulls to freezing, the introduction of a variety of extending media, and new procedures for packaging and freezing added new variables (16). Generally, the nonreturn rates, used as a measure of fertility, were similar when 30×10^6 to 40×10^6 total sperm were used for AI, but results varied when the sperm numbers used were 20×10^6 total sperm or fewer (1, 4, 8, 9, 14, 20, 22). Hunter (10) reported little difference in nonreturn rate when 10×10^6 or 20×10^6 total sperm were used for AI by more skillful inseminators, but a 10-percentage unit difference, associated with sperm numbers, was found when semen was used by less skillful inseminators.

In the area served by Genex Cooperative, Inc. (Ithaca, NY), professional inseminators were used to conduct several fertility trials with different numbers of sperm, while consistently using the same processing procedures from the time of semen collection to AI. The objective of the studies reported here was to estimate accurately the relationship between number of the sperm packaged in 0.5-ml straws and fertility (nonreturn rate) under well-controlled conditions.

MATERIALS AND METHODS

Bull Semen

All semen was collected from Holstein bulls in regular AI service at Genex Cooperative, Inc. Following sexual preparation, two ejaculates usually were obtained from each bull on any single morning, processed separately during initial extension, cooled to 5°C, and then combined for final processing and freezing. Occasionally, a third ejaculate was collected

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after an interval of at least 30 min from the previous semen collection.

Extender and General Semen Processing

Homogenized whole milk (**WM**) was heated to 95°C for 10 min, cooled, and filtered; antibiotics were added (5, 13), followed by the addition of glycerol as the cryoprotectant. Two fractions of WM were prepared daily: fraction A was heated WM without glycerol, and fraction B contained 14% glycerol (vol/vol). Before use, fraction A was placed in a waterbath at 35°C, and tubes of collected semen were placed in the same waterbath.

Semen was subsampled, as collected, to conduct various tests of semen quality. The sperm concentration was estimated by optical density, using a calibrated spectrophotometer. The percentage of motile sperm was estimated at 37°C with the aid of a television monitor connected to a microscope.

The remainder of the semen was extended 1:4 (vol/vol) with fraction A of the WM extender, placed in a water jacket at 35°C, and cooled for 3 h to 5°C. The cooled, partially extended semen was further extended with cold WM, fraction A, to one-half the final volume. Later, an equal volume of WM containing 14% glycerol was added by the drop method.

Extended semen was packaged in 0.5-ml straws, sealed, and frozen within 4 h after glycerol addition. The cooling rate was approximately −15°C/min from +5 to −100°C; after cooling, straws were transferred to liquid nitrogen. Details about sperm numbers per straw and other experimental details are given for each experiment. All straws were coded to identify the control and experimental semen for researchers. The code was recorded by the inseminating technicians, but the specific treatment that was represented by each code was unknown to them.

The straws from each collection and the treatment of semen were randomly distributed over a large group of technicians for AI relatively soon after processing. Thus, possible sources of variation in the field were distributed as equally as possible across treatments. Rate of semen use depended on semen sales from each bull, but the rate was similar for the control and treated portions of semen from each bull. A subset of straws was retained for quality control and for additional studies on the percentage of motile sperm after freezing and thawing.

Experiment 1

Twenty bulls in regular AI service were assigned in groups of 4 for semen collection. On a particular day, each bull had semen extended to yield 20×10^6, 25×10^6, 30×10^6, or 40×10^6 total sperm per straw. With one or more groups of bulls ejaculated on the same day, all semen treatments were represented each day that semen was collected. The procedure was continued until all bulls were represented in all four treatments; a minimum of 600 straws of frozen semen per subclass was available for breeding. The 20 bulls were ejaculated on 136 occasions to provide sufficient sperm; most of the collections beyond 80 (20 bulls × 4 treatments) were required to fulfill the requirements for the treatments using 30×10^6 and 40×10^6 sperm.

The initial concentration of sperm per milliliter, estimated by spectrophotometry, was checked with a Coulter Counter (Coulter Electronics, Hialeah, FL). An aliquot of semen was appropriately diluted with counting fluid to provide statistically accurate counts with the counter.

The randomized block design was analyzed statistically by the general linear models procedure of SAS (19); bulls were regarded as random blocks, and treatments were fixed. The sampling error consisted of multiple semen collections within bulls and treatment. Treatment differences were considered to be statistically significant at $P \leq 0.05$.

Experiment 2

This experiment was a split-plot design. Semen from each of 10 bulls was extended so that the straws prepared from each semen collection contained a planned number of total sperm: 12×10^6, 16×10^6, or 20×10^6. Semen from all 10 bulls was collected on four occasions, providing a sampling error estimate for the analysis of variance. Again, bulls were regarded as random, and treatments were regarded as fixed.

A subset of straws was retained to estimate by a flow cytometric procedure (15) the actual number of sperm that were distributed to the straws for each treatment of each semen collection. The sperm were exposed to both rhodamine R123, a dye sequestered by functional mitochondria, and propidium iodide, which stains dead cells. Thus, both total sperm and viable sperm were estimated.

Experiment 3

This experiment was a randomized block design similar to that used in Experiment 1, and the statistical analysis followed the same plan as for Experiment 1. Ten bulls were used to extend semen from different collections to 10×10^6, 13×10^6, 16×10^6, or 20×10^6 total sperm per straw. The number of bulls was

limited to the highest demand bulls so that the semen would be used rapidly in the field. The ejaculates were not split, because a four-way split was difficult to handle technically in the processing laboratory, and the number of straws available for each treatment subclass within ejaculates would be too few for adequate distribution across a large group of inseminators and herds. On average, 750 straws would be expected to be processed per collection from each bull; more or fewer might be processed depending on the sperm number treatment. At least 7500 services per treatment were desired in order to detect differences in fertility ≥2%.

Semen was collected from all bulls at least twice for each sperm number treatment. At least 50% of the experimental semen was expected to be used for cows at the first AI service. Approximately 60,000 straws were produced ($10 \times 4 \times 2 \times 750$), and at least 30,000 straws were likely to be used for the first AI.

RESULTS

Results for all three experiments are summarized in Table 1. There was no difference in fertility associated with sperm numbers ($P > 0.05$). However, fertility tended to decrease as sperm number decreased below 20; in Experiments 2 and 3, the probability values for treatment were $P = 0.06$ and $P = 0.10$, respectively. Bulls were the major source of variation in all experiments. The range in fertility among bulls in Experiments 1, 2, and 3, respectively, was 64 to 76%, 66 to 76%, and 66 to 75%. There was no evidence of an interaction between bull and treatment.

The estimated percentage of progressively motile cells after thawing in warm water at 35°C was used to estimate the number of motile sperm that was available per AI. In Experiment 1, 52 to 54% of the sperm were estimated to be progressively motile, resulting in the motile sperm values being about one-half that of total sperm numbers (Table 1). Facilities and procedures were similar in Experiments 2 and 3. In Experiment 2, the percentage of progressively motile sperm cells across treatments estimated visually was 49 to 51% and, by flow cytometry, ranged from 50 to 56%. The total number of sperm in the straws estimated by flow cytometry for the three treatments was 19.8×10^6, 15.8×10^6, and 11.4×10^6. Thus, the planned sperm number was essentially achieved. There was no correlation between the estimated percentage of motile sperm in each treatment within bull subclass and fertility.

In Experiment 3, the estimated percentage of motile sperm in the frozen and thawed semen ranged from 46 to 48% for the four treatments, which resulted in good correspondence between results of the last two experiments in the estimated number of motile sperm available for AI in treatments that had approximately equivalent total sperm per straw. The fertility obtained with the 10 bulls at the four sperm numbers was not correlated with the percentage of progressively motile sperm after freezing and thawing ($r = 0.04$; $P > 0.05$). Also, the number of motile sperm per straw was not correlated with fertility.

TABLE 1. Nonreturn rates with different numbers of sperm frozen in whole milk glycerol extender packaged in 0.5-ml straws.

Experiment	First service	Sperm number		Nonreturn rate[1]
		Total	Motile	
	(no.)	($\times 10^6$)		(%)
1	8037	20	10.4	69.8
	7850	25	13.5	70.0
	7547	30	16.2	70.1
	7965	40	21.6	70.1
2	6199	12	6.1	70.2
	7917	16	7.8	72.4
	6081	20	10.2	70.8
3	9805	10	4.6	70.5
	8579	13	6.2	72.2
	10,024	16	7.7	73.1
	10,482	20	9.4	71.5

[1]Nonreturn rates within experiments did not differ ($P > 0.05$).

DISCUSSION

The usual goal in AI is to make available as many breeding units as possible from bulls of superior genetic merit without affecting fertility appreciably. The provision of enough sperm per breeding unit, but not more than are needed, is a key in accomplishing this goal. A curvilinear relationship between the sperm numbers that were inseminated and nonreturn rate was graphically portrayed for experiments with liquid semen by Bratton et al. (3). Later, this general relationship was discussed by Salisbury et al. (18).

Experiments have been reported from various countries that involved skim milk, homogenized whole milk, egg yolk-Tris, egg yolk-citrate, egg yolk-TES-Tris, and various other combinations with egg yolk, all with cryoprotectant and antibiotics (1, 2, 4, 6, 7, 8, 9, 10, 11, 14, 21, 23). Also, different packaging was used (0.50-ml ampules and 0.25-ml and 0.5-ml straws), different freezing rates were employed, and different field conditions existed. Thus, it is not possible to identify specific causes of differences among studies regarding nonreturn rates obtained. All studies referenced used nonreturn rates to esti-

mate fertility, although other estimation procedures have been suggested (12).

Within studies, the published results indicate that increasing total sperm numbers beyond 20×10^6 sperm per breeding unit did not improve fertility. Downward trends were 1.2% (4) when sperm numbers were decreased from 36×10^6 to 18×10^6 and 2.5% (9) when sperm numbers were decreased from 35×10^6 to 12×10^6, but differences were not significant ($P > 0.05$). Schenk et al. (20) reported that using 11×10^6 total sperm per AI resulted in a 4% lower nonreturn rate ($P < 0.05$) than was achieved using 15 to 22×10^6 total sperm. Recently, Kommisrud et al. (11) reported that fertility did not differ when 18×10^6 and 12×10^6 total sperm were used, but 15×10^6 total sperm gave a lower nonreturn percentage of 1.2% ($P < 0.05$). This apparent inconsistency is a reflection of the random variation that is associated with the binomial nature of pregnancy. Gérard and Humblot (7) found a difference of 3.5% in nonreturn rate ($P < 0.05$) when the total number of sperm per straw was reduced from 16×10^6 to 8×10^6. Pace et al. (14) reported nonreturn rates of 69.0, 67.3, 67.1, and 64.1% ($P < 0.05$) when total sperm per breeding unit was 18.2, 14.8, 9.1, and 4.7×10^6. The postthaw motile sperm in the various studies ranged from about 35% to nearly 60%.

Several reports indicate that low sperm numbers become more of a concern when fertility of bulls is low (11, 22, 23) or when semen is used by less effective inseminators (10, 14). However, in some of those studies, bulls were retrospectively assigned to fertility groups based on the experimental results. Comparisons in those studies are biased because of incomplete repeatability, and independent sets of fertility data should be used to rank bulls.

In our studies, we made a special effort to check that planned sperm numbers were achieved. The actual value in Experiment 2 for the percentage of nonreturns was nonsignificantly higher ($P > 0.05$) in the treatment with 16×10^6 total sperm than in the treatment with 20×10^6 total sperm; this random difference was opposite the result obtained by Kommisrud et al. (11). There was a nonsignificant ($P > 0.05$) difference of 2.6% between 16×10^6 and 10×10^6 total sperm in Experiment 3. However, the nonreturn rate difference between use of 20×10^6 and 10×10^6 sperm was only 1%. This small decline was not due to any particular bull because there was no interaction between bull and treatment.

Statistical significance was approached for fertility differences between 16 and 12×10^6 sperm in Experiment 2 and 16 and 10×10^6 sperm in Experiment 3. Thus, these data tend to support the common practice of AI organizations to process most frozen semen to ensure more than 10×10^6 total sperm per breeding unit or straw. At the same time, the processing of breeding units from genetically superior, high fertility sires, with no more than 10×10^6 total sperm, could have beneficial genetic effects without reducing fertility when these sperm replace sperm from bulls of lower fertility and lower genetic merit.

These studies collectively demonstrate that, under good conditions of collecting, processing, and inseminating semen, high fertility is maintained with 10×10^6 total sperm (about 5×10^6 motile sperm) per breeding unit. Additional sperm are unlikely to improve fertility appreciably. However, under conditions in which fertility tends to be low, such as with inexperienced inseminators, fertility would not be expected to be as high as was obtained in the experiments reported here.

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Attempts on freezing the Greylag (*Anser anser* L.) gander semen

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Abstract

Semen of Greylag (*Anser anser* L.) ganders was frozen according to a method previously elaborated by the authors for freezing the White Koluda gander semen.

Semen was collected from five to eight Greylag ganders, twice a week during three succeeding reproductive cycles, by dorso-abdominal massage. Semen samples were diluted in the ratio of 1:1 or 2:1 (two parts semen: one part diluent) with EK diluent, supplemented by 6% DMF, equilibrated and pre-frozen to −140 °C at a rate 60 °C/min, before being transferred into liquid nitrogen container. Semen samples thawed in a water bath of 60 °C were used for twice a week insemination in a volume of 200 μl. Three Greylag and three White Koluda geese were involved in frozen-thawed semen fertilizing ability test.

The reproductive cycle of wild geese lasts usually about 6–7 weeks. The ejaculate volume (30–140 μl) and sperm concentration (10×10^6 to 150×10^6 ml^{-1}) are much lower than these of domestic ganders, but spermatozoa morphology is similar, particularly while compared to 1-year-old White Koluda ganders semen. There are about 90% of live spermatozoa and about 30% of live morphologically normal cells in Greylag gander fresh semen.

The Greylag gander spermatozoa susceptibility to cryopreservation procedure is as high as in domestic gander'. Dilution ratio 2:1 resulted in higher number of live spermatozoa, which withstood cryoinjury stress. In relation to fresh semen about 60% of spermatozoa remained intact (on the basis of light microscope examination) in the frozen-thawed semen. Insemination of frozen-thawed semen resulted in 37.5% of fertile eggs in Greylag and 25.0% in White Koluda geese. Low fertility rate was caused by an insufficient number of live normal spermatozoa used for insemination (about three million in every dose).

Keywords: Goose; Greylag geese; Semen freezing; Spermatozoa morphology; Fertility

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1. Introduction

Greylag goose (*Anser anser* L.) is the only species of wild geese that nest in Poland. Since for several years these birds have been strictly protected the number of nesting geese and flock population is rather stable. Nevertheless industrial development, intensification of agriculture and environmental pollution might be dangerous and cause the goose population decline in the nearest future. Therefore, attempts on semen long-term storage in liquid nitrogen (LN_2), as a way of gene pool preservation seems to be very desirable.

The pilot experiments carried out at Agricultural University of Wrocław (Poland) on possibilities of Greylag geese taming and handling (ganders for semen collection, geese for insemination) showed that proper treatment allows to obtain semen from males and fertile eggs from females (Chełmońska et al., 1993).

Further experiments on introducing the wild goose in commercial breeding program, mainly in order to improve a slaughter value and meat quality of domestic geese, by means of artificial insemination technique, resulted in obtaining healthy gosling hybrids from reciprocal crossbreeding of Greylag and domestic goose White Italian (*Anser anser* L.) (Chełmońska and Chrzanowska, 1996; Chrzanowska and Chełmońska, 1997, 2000). The evaluation of body weight and zoometrical measurements of the reciprocal hybrids revealed that their slaughter value was improved in comparison with parents. The body weight of hybrids at the age of 8 weeks was lower but the chest measurement was higher by 3 cm in comparison with White Italian goose (Chełmońska et al., 1995) and their hybrids with Polish regional goose varieties (Mazanowski and Chełmońska, 2000). Also the content of subcutaneous fat in both reciprocal hybrids (15%) was significantly lower than in carcass of White Italian goose (23%).

The greatest problem connected with wild goose reproduction in captivity and consequently limiting wild and domestic goose hybrids production on a commercial scale is, except low semen quantity and quality, a very short reproductive cycle of wild geese. The reproductive cycle of Greylag goose lasts usually 6 weeks (from middle of March to the end of April) (Chrzanowska and Chełmońska, 1997) while one of domesticated goose—6 month (from January to the middle of June) (Chełmońska, 1972; Łukaszewicz, 2000, 2002b; Rosiński et al., 1986). Moreover, the Greylag goose is very watchful and shy, therefore the onset of reproductive cycle depends, among the others, on environmental temperature and goose immediate surrounding. The differences in duration of the reproductive cycles do not allow hybrids production via artificial insemination with the fresh semen within the reproductive cycle of domestic goose. The possibility of semen storage in liquid nitrogen for unlimited period would partly solve this problem and also could be helpful in wild goose gene pool preservation.

Several successful experiments on freezing the semen of wild birds species, resulting in progeny obtained after artificial insemination with frozen-thawed semen, have been hitherto done (Blanco et al., 2000; Chalah et al., 2001; Gee, 1995; Gee et al., 1984, 1993; Gee and Sexton, 1990; Knowles-Brown and Wihart, 2001; Penfold et al., 2001; Sexton and Gee, 1978). Nevertheless, because of species dependent differences in fresh semen quality and spermatozoa susceptibility to freezing, there exist no single comprehensive cryopreservation method which could be effective for all birds species (Blanco et al., 2000; Holt, 2000; Polge, 1980).

The authors elaborated the method of freezing the semen of White Koluda (formerly known as White Italian) ganders, descended from Greylag goose. The application of the method resulted in over 90% of fertile eggs after twice a week goose insemination with about 10 million of live morphologically normal frozen-thawed spermatozoa (Łukaszewicz, 2002b).

The objective of the present experiments was to examine if the cryopreservation method described for White Koluda gander semen (Łukaszewicz, 2001a,b) will be equally effective for Greylag gander semen.

2. Materials and methods

2.1. Birds

The experiments were carried out at the Department of Poultry Breeding, Agricultural University of Wrocław, during three succeeding wild goose reproductive cycles (Experiments I–III). Number of adult ganders subject to semen collection varied from 5 (Experiment I) to 8 (Experiments II and III). In order to evaluate the fertilizing ability of frozen-thawed semen, in the last cycle (Experiment III) three Greylag and three White Koluda geese were used.

One month prior to the beginning of every reproductive cycle, the ganders were separated from females and transferred to individual cages (70 cm × 95 cm × 85 cm) which were situated within the same pen as females. The previous behavioural observations showed that visual and vocal contact with females stimulates ganders to better response to massage and semen production. At the and of the reproductive cycle the males were removed from cages and kept together with females. Geese subject to fertility tests were housed in open-air pen with free access to water basin.

All birds were kept in unheated housing, under natural light and fed ad libitum with commercial feed for breeding geese. The mixture contained 11.1 MJ of metabolise energy and 140 g of crude protein per kilogram.

During the three reproductive cycles there were no changes in husbandry techniques and feeding regime. The weather conditions constituted the only uncontrolled factor.

2.2. Semen collection, evaluation and methods of treatment

Every year, about 3 weeks before semen collection, all ganders were trained for handling, massage and attempt to obtain an ejaculate. Pooled semen was collected twice a week by dorso-abdominal massage. During semen collection particular care was taken to minimise contamination of semen with uric or faecal. In order to maximise the semen quality and quantity, the collection was performed always under the same conditions (environment, time, persons and massage procedure). During one reproductive cycle 10–14 semen collections were performed from every male.

In the freshly collected semen the following parameters were examined: pooled ejaculates volume, consistency, colour, blood or faecal contamination, spermatozoa motility, concentration and morphology. Motility and morphology of frozen-thawed spermatozoa

were evaluated in every experiment. Additionally, in the Experiment III, fertilising ability of frozen-thawed semen was examined.

Integrity and morphology of the spermatozoa (n = 300 cells per slide) were examined in nigrosin–eosin smears and evaluated at 1250× under a light microscope (Jenaval, Carl Zeiss, Jena, Germany). Spermatozoa were attributed to seven categories: six of them, named "total live", were as follows: (1) morphologically normal (typical spindle-shaped head and well-marked acrosome); (2) bulb-head; (3) crooked-neck; (4) midpiece deformed (swelling, ragged or lack of midpiece); (5) spermatids (immature forms); (6) spermatozoa with other deformities (not included in any of the previous category). Dead spermatozoa stained by eosin were indicated as category 7. The results of morphological evaluation were expressed as the percentage of particular categories of spermatozoa (300 cells = 100%).

The concentration of spermatozoa was calculated in a hemocytometer with the use of eosin—3% NaCl solution; motility was evaluated under light microscope. Positive movement of individual spermatozoa was assessed in a scale ranging from 10 to 100% of motile cells (Bielański, 1972).

For better evaluation and comparison of fresh semen quality, especially with the respect to the number of spermatozoa potentially capable of ovum fertilization, the semen quality factor (SQF), calculated according to following pattern was applied:

$$\mathrm{SQF} = \frac{\text{sperm concentration per ml} \times \text{ejaculate volume (ml)} \times \text{live normal spermatozoa (\%)}}{100\%}$$

To understand better the influence of freezing process on spermatozoa morphology, the relative changes (RCH) were calculated (in every spermatozoa morphological category of spermatozoa, the percentage of cells that survived freezing was compared with an adequate category in fresh, adopted as 100%).

Freshly collected, clean semen samples were diluted at room temperature with EK diluent (Łukaszewicz, 2001a, b) in 1:1 ratio (in Experiments I and II) and 2:1 (two parts of semen: one part of diluent) in Experiment III and frozen according to method already described (diluted semen was equilibrated for 15 min, mixed with 6% (v/v) of dimethyl-formamide, aspirated into 0.25 ml plastic straws, placed in a computerised freezing unit "Minidigitcoll 1400" (IMV ref. ZB280) and after 5 min of further equilibration the straws were pre-frozen to −140 °C, at a rate of 60 °C/min, then plunged into liquid nitrogen (−196 °C) container.

Semen samples were thawed by placing the straws for 4–5 s into water bath (60 °C).

2.3. *Fertilising ability test of frozen-thawed semen*

Fertilizing ability test of frozen-thawed Greylag gander semen was performed in the Experiment III only, both, for Greylag and White Koluda geese. Females were inseminated twice a week with a dose of 200 μl of thawed semen. Ten inseminations of White Koluda and six of Greylag geese were performed. Semen was deposited intravaginally by the finger-guided method, using the plastic pipettes (IMV, U 212) and the inseminating pistol (IMV, U 695). In every semen dose, the spermatozoa concentration and morphology was evaluated prior to insemination, in order to estimate the number of live morphologically normal spermatozoa introduced in the inseminated semen volume.

Fecundity results (number of set eggs, fertility, hatchability of set and fertile eggs) were determined for eggs collected daily, from Day 2 after the first insemination to Day 4 after the last insemination. The eggs were set weekly to the Type C 82 multistage incubators (Agraria, Gostyń, Poland). Five egg sets for White Koluda and three for Greylag goose were made. Eggs were candled on Days 9 and 24 of incubation. All fertile eggs were incubated up to the hatching and hatchability results were determined. The results were expressed as the average data obtained for all eggs.

3. Statistical analysis

The differences in number of particular categories of spermatozoa in the fresh and frozen-thawed semen were analyzed with ANOVA and Duncan's multiple range test (SAS system, General Linear Models Procedure).

4. Results

The first ejaculates were obtained after 2–3 weeks of training, i.e. from the second half of February, almost in every reproductive cycle. The last ejaculates were collected at the end of March or up to the middle of April. Individual differences in gander' responses to massage, development of copulatory organ and ejaculate quality were observed. Some ganders responded with semen ejaculation after two to three initial attempts while the other had to be trained for further 2–3 weeks before semen production occurred. Also during the reproductive cycle the individual differences in reaction to massage were evident. In case of some males the longer massage and a gentle pressure had to be applied around the cloaca region in order to extrude the phallus.

Quantity and quality of the fresh semen collected during three reproductive cycles were similar and existing differences were statistically not significant (Table 1, Fig. 1).

Single ejaculate volume varied from one drop (about 30 μl) to 140 μl, while the spermatozoa concentration in pooled semen collected in three reproductive cycles varied between 10×10^6 and $150 \times 10^6\,ml^{-1}$. The average data for fresh semen collected in evaluated cycles are given in Table 1.

No significant differences were detected in spermatozoa morphology in three succeeding reproductive cycles. The total number of live spermatozoa was high and rather stable, varying from 82 to 96%, but live morphologically intact cells constituted only from 15 to 50%. In the fresh semen the macrocephalic spermatozoa were the most frequent and ranged from 17 to 59%. Also the quantity and quality of ejaculates expressed as semen quality factor (SQF) were comparable in the evaluated cycles, but had a very low values. A decrease in semen quality along with the reproductive cycle progress could have been observed in each Greylag reproductive cycle (Fig. 1).

Freezing process had a harmful effect both, on spermatozoa motility and morphology. Percentage of motile cells decreased from about 60% in fresh semen to 30–40% in frozen-thawed semen. The movement of thawed spermatozoa was slower compared to the fresh semen, moreover an abnormal, pulsating movement was observed in about 20% of

Table 1
Characteristics of the fresh and frozen-thawed semen of Greylag ganders (*Anser anser* L.) and relative changes in spermatozoa morphology between fresh and frozen-thawed semen, in succeeding three reproductive cycles

Evaluated traits	I cycle—Experiment I			II cycle—Experiment II			III cycle—Experiment III		
	Fresh semen	Thawed semen	RCH[a]	Fresh semen	Thawed semen	RCH	Fresh semen	Thawed semen	RCH
Number of ganders	5	–	–	8	–	–	8	–	–
No of semen collection or freezing procedures	14	8	–	12	12	–	10	10	–
Pooled ejaculates volume (ml)	0.38 ± 0.20	–	–	0.55 ± 0.20	–	–	0.56 ± 0.17	–	–
Ejaculate/male (ml)	0.075 ± 0.04	–	–	0.07 ± 0.03	–	–	0.07 ± 0.02	–	–
Spermatozoa concentration ($n \times 10^6$ ml^{-1})	35.36 ± 29.12	–	–	81.25 ± 28.69	–	–	77.00 ± 35.92	–	–
SQF/male[b]	1.06 ± 1.1	–	–	1.64 ± 1.00	–	–	1.47 ± 0.88	–	–
Spermatozoa categories (%)									
Total live	91.26 ± 3.59	26.96 ± 7.10	29.18 ± 7.53	93.28 ± 1.87	61.61 ± 10.95	66.08 ± 11.79	90.27 ± 4.65	62.07 ± 11.10	69.07 ± 13.22
Live morphologically normal	31.17 ± 8.90	8.42 ± 1.81	25.55 ± 5.15	28.61 ± 6.97	10.69 ± 2.23	39.76 ± 13.40	26.17 ± 5.60	14.90 ± 4.82	59.67 ± 22.07
Macrocephalic	35.60 ± 9.13	12.54 ± 5.43	39.56 ± 21.16	28.69 ± 5.40	24.36 ± 6.40	85.58 ± 19.53	23.57 ± 6.30	19.77 ± 4.43	91.57 ± 40.61
Bent-neck	10.24 ± 3.08	3.83 ± 1.89	95.16 ± 31.96	18.00 ± 4.41	18.25 ± 9.22	111.28 ± 70.84	20.60 ± 5.82	20.77 ± 8.42	100.30 ± 24.32
Midpiece deformed	7.26 ± 2.23	2.17 ± 1.50	26.55 ± 17.9	6.94 ± 2.33	4.75 ± 2.30	85.28 ± 72.6	6.80 ± 2.94	3.80 ± 2.55	61.1 ± 45.4
Spermatids[c]	5.79 ± 2.72	–	–	8.44 ± 2.57	–	–	7.23 ± 2.70	–	–
Other deformities	1.21 ± 1.42	–	–	2.67 ± 1.15	3.56 ± 2.83	128.7 ± 70.3	5.50 ± 3.81	2.83 ± 1.21	75.9 ± 93.2

[a] RCH: relative changes, percent of spermatozoa which withstood after freezing, in relation to fresh semen adopted as 100%.
[b] Semen quality factor = (sperm concentration ($n \times 10^6$ ml^{-1}) × ejaculate volume/male (ml) × live morphologically normal spermatozoa (%))/100%.
[c] Spermatids: immature cells were counted only in the fresh semen.

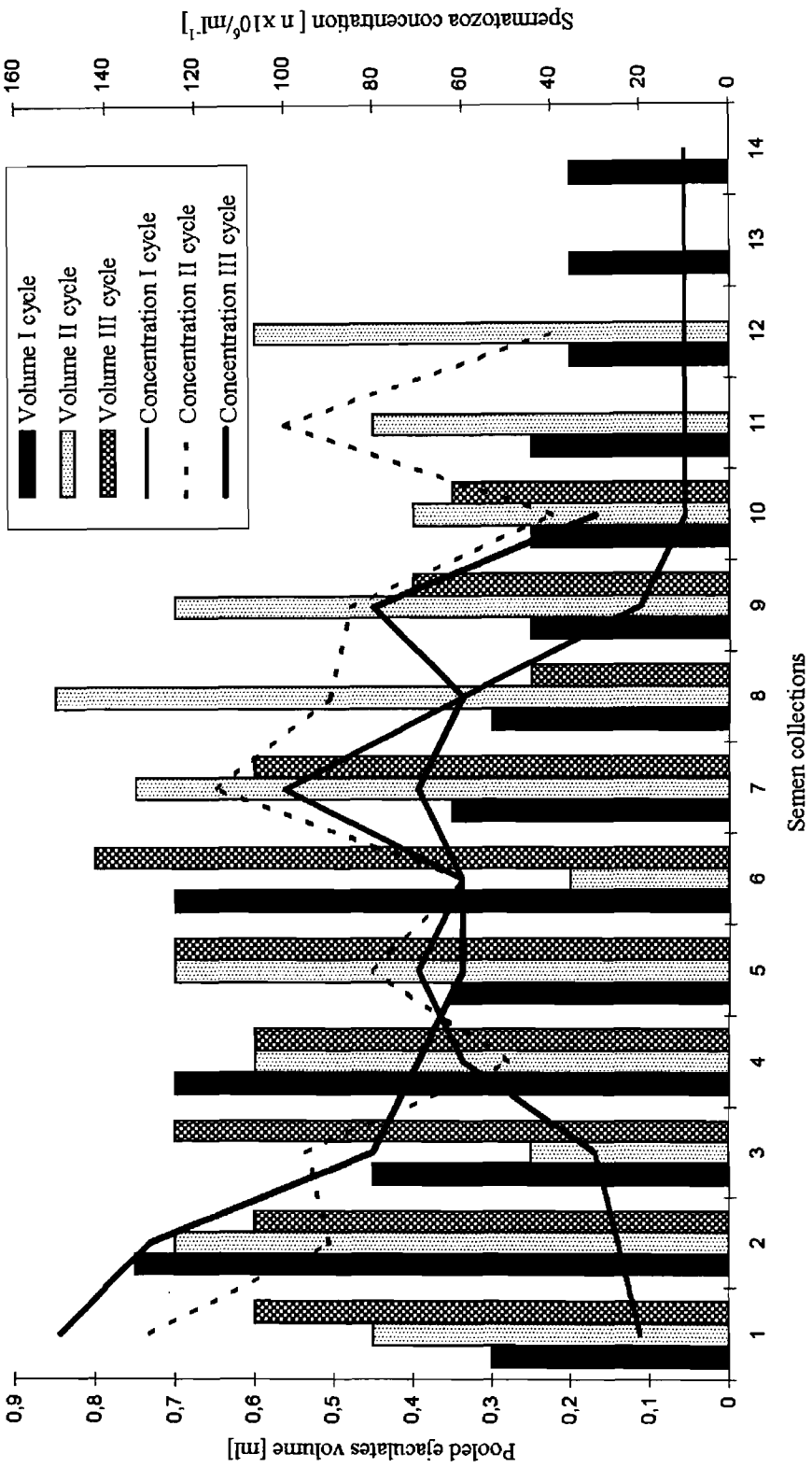

Fig. 1. Ejaculates volume and spermatozoa concentration in Greylag gander semen manually collected in three reproductive cycles.

Table 2
The fecundity results of Greylag and White Koluda geese inseminated twice a week with 200 μl of frozen-thawed Greylag gander semen (means ± S.D.)

Evaluated traits	Greylag (three geese)	White Koluda (three geese)
Number of inseminations	6	10
Total live sperm in a dose (million)	6.0	6.0
Normal sperm in a dose (million)	3.0	3.0
No of set/fertile eggs[a]	8/3	48/12
Fertility (%)	37.5	25.0
No of dead embryos	–	–
No of hatched goslings	3	12
Hatchability (%) of set eggs	37.5	25.0
Hatchability (%) of fertile eggs	100.0	100.0

[a] Data for eggs collected from the 2nd day after first to 4th day after the last insemination.

live cells. For almost all categories of spermatozoa, with the exception of macrocephalic and bent-neck spermatozoa in Experiments II and III (Table 1), the differences between fresh and frozen-thawed spermatozoa were statistically significant ($P \leq 0.01$).

The survival rate of frozen-thawed spermatozoa was correlated with the fresh semen dilution ratio. Lower dilution rate (2:1 versus 1:1) better influenced spermatozoa resistance to cryoinjury stress (Table 1). In Experiment III, where fresh semen was diluted in 2:1 ratio, the percentage of total live and live morphologically undamaged spermatozoa (evaluated under light microscope) which withstood freezing-thawing procedure was significantly higher in comparison with 1:1 ratio (Experiments I and II, Table 1). The differences are even more evident when freezing effect is expressed as relative changes (RCH) between fresh and frozen-thawed semen. In frozen-thawed semen in Experiment III, live morphologically normal spermatozoa, i.e. these which theoretically posses the highest fertilizing potency, constituted on average nearly 60% of the number of adequate spermatozoa in the fresh semen, while in Experiments I and II there were 26 and 40%, respectively.

The fertility rates of eggs laid by wild Greylag and domestic White Koluda geese inseminated with frozen-thawed were low, and averaged at 37.5 and 25.0%, respectively, however the number of inseminated live undamaged spermatozoa was also very low—about 6 million cells per week (Table 2). Therefore, on the basis of the obtained result it is impossible to state that cryopreservation process significantly decreased the fertilizing ability of spermatozoa.

5. Discussion

The Greylag goose (*Anser anser* L.) is recognised as one of two ancestors of numerous domestic goose breeds. Most of geese bred nowadays in Europe on a commercial scale are probably derived from this wild species (Crawford, 1990). The reproductive cycle of wild goose is considerably shorter than this of domesticated goose, its length both, in birds living in freedom and kept in captivity depends, among the other, on environmental conditions and usually lasts no longer than 6 weeks. Ambient temperature and any stress influence a reproduction behaviour and reproductive efficiency of wild goose (Lorenz, 1963).

Individual differences in Greylag gander's reaction to massage, size of copulatory organ and quality of ejaculates were similar to these observed among domestic White Koluda ganders (Łukaszewicz, 2002b).The volume of ejaculates (from 30 to 140 μl) and spermatozoa concentration (10×10^6 to 150×10^6 ml^{-1}) of manually collected semen from Greylag ganders are several times smaller than these of domestic White Koluda ganders. The motility and morphology of wild and domestic gander spermatozoa are similar, but far worse comparing to semen of any other poultry species (Chełmońska and Łukaszewicz, 1995; Łukaszewicz, 2000, 2002b). The number of the most desirable form of spermatozoa—live, morphologically normal cells in Greylag gander semen reached about 30% and was similar to one was observed in 1-year-old White Koluda ganders. In older, 2–4 years old ganders this characteristics is usually higher but only sporadically exceeds 50% (Łukaszewicz, 2001a, 2002b). In the fresh semen of Sandhill Crane Gee and Temple (1978) observed 57% (±18.4%) of undamaged spermatozoa on average. The differences in fresh semen quality of wild and domestic gander are more evident when Semen Quality Factor is compared. It reached on average 1.5 for Greylag and from 111.5 to 1.1 for White Koluda gander semen (Łukaszewicz, 2002a,b).

Nevertheless, despite significantly lower fresh semen quality of Greylag ganders, the spermatozoa susceptibility to cryopreservation procedure seems to be as high as one of White Koluda gander.

The applied cryopreservation procedure resulted in 15% of live morphologically intact spermatozoa on average, in the thawed semen, which constitutes about 60% of spermatozoa number in the fresh semen (so called relative changes—RCH). For White Koluda gander semen this data varied from 28 to 53%, depending on the age of male, phase of the reproductive cycle and individual male properties (Łukaszewicz, 2002b). A similar susceptibility to cryopreservation procedure (average 43 of RCH value) was stated for Aleutian Canada goose (Gee and Sexton, 1990). In fresh and frozen-thawed semen there were 92.9 ± 2.5 and 46.7 ± 7.8% of live spermatozoa. In the experiment carried out by Tai et al. (2001), the number of live spermatozoa decreased due to freezing procedure from 83% in a fresh gander semen to 27 or even 7.3% after thawing (i.e. the RCH values were 22 and 6.1, respectively).

The beneficial effect of lower dilution rate of Greylag gander fresh semen on spermatozoa susceptibility to cryopreservation procedure have been observe also for domestic White Koluda gander semen, characterised by low spermatozoa concentration (Łukaszewicz, 2001b; 2002b).

Fertility of eggs obtained from geese inseminated with frozen-thawed Greylag gander semen was low, but considering a very low number of cells being introduced into oviduct, it could be concluded that fertilizing ability of thawed spermatozoa was rather good. The authors have already proved, that contrary to the chicken, the fertilizing ability of frozen-thawed gander spermatozoa seems to be affected by freezing in a smaller degree. Fertility after insemination with frozen-thawed gander semen depends mainly on insemination frequency and number of inseminated live, morphologically normal spermatozoa. After insemination with 9.5 or 18 million of live normal spermatozoa per week of frozen-thawed White Koluda gander semen, fertility was 84.8 and 93.0%, respectively (Łukaszewicz, 2002b).

The fertility rates amounting 37.5 and 25.0%, respectively, for Greylag and White Koluda geese inseminated with about six million of live frozen-thawed spermatozoa of wild Greylag

gander seem to be quite satisfactory. Even in naturally mated goose flocks fertility varied from 48 to 79% (Nitsan et al., 1988). Grunder and Pawluczuk (1991) obtained 54% fertile eggs inseminating the geese one a week with 14 million. It can be expected that enlarged number of inseminated cells will lead to higher, more effective fertility rate.

6. Conclusion

The percentage of live morphologically normal spermatozoa in frozen-thawed Greylag gander semen indicates that the cryopreservation method worked out for White Koluda (*Anser anser* L.) gander semen is also effective for wild Greylag gander semen. This result permits to expect that this method can be useful in freezing semen of other goose species derived from *Anser anser* L. and can serve as their germ plasm cryopreservation.

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Low Dose Insemination in the Sow – A Review

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Contents

Artificial insemination (AI) in pigs has been established for about four decades but ejaculates are still used insufficiently. Higher demand of semen for AI and new techniques that involve low sperm concentration require the optimization of insemination protocols. Based on the knowledge of the physiology of sperm transportation and events in the female genital tract prior to fertilization, new strategies are under development to minimize sperm losses. One goal is to deposit the semen into the uterine horn rather than into the proximal cervix. It was shown that the minimal number of spermatozoa necessary for surgical AI at the utero-tubal junction (UTJ) were at least 1×10^6 diluted in 0.5 ml of a special extender. Artificial insemination into the distal part of the uterine horn required about 1×10^7 million sperm in 20 ml of extender. Meanwhile, first insemination devices for non-surgical intra-uterine AI are commercially available. Using similar sperm concentrations as for surgical AI, non-surgical uterine insemination did not differ significantly from control inseminations in terms of pregnancy rate and litter size. With respect to the fertilizing capacities of their ejaculates, boars have to be selected more strictly for sperm quality parameters as most of the compensatory effects of sperm cells disappear in maximally extended semen samples.

Introduction

As the usage of artificial insemination (AI) in pigs has increased rapidly in recent years, the demand for semen from genetically superior boars has grown and can only be satisfied by using more boars for collection. This reduces selection efficiency and raises both capital and labour costs. Compared with other species, only a limited degree of dilution is currently possible with boar ejaculates (Ciereszko et al. 2000) because even with special extenders 2–3.5 billion spermatozoa are needed in each 80–100 ml dose (Colenbrander 1991). Although experiments were performed in the 1950s and 1960s to optimize AI strategies in pigs, no real breakthrough was ever achieved. However, as commercial interest is now focused on new biotechniques like AI with sexed semen and transgenic sperm cells, the discussion has reemerged as to what might be the optimal sperm concentration for pig AI. Testing is complicated in comparison with bovine AI by the fact that both pregnancy rate and litter size are factors in successful porcine AI.

The comparatively large dose of semen is necessary because boars are 'intra-uterine inseminators'. Their normal ejaculate volume is much larger than in ruminants and their ejaculate is less concentrated. The sow is a multiparous animal with long uterine horns and sperm travel a long way from the site of insemination to the oviductal ampulla to meet the oocytes. The growth of follicles and the time of ovulation cannot be verified directly by rectal palpation. Ultrasonography may be a solution in the future, but so far only secondary signs of oestrus are used as indicators to determine the time of insemination. Other events in the cycle are estimated from this imprecise time point and vary between animals, breeds, farms and other circumstances. Therefore, it is normal practice to inseminate more than once per cycle with high sperm numbers to achieve fertilization (Reed 1982; Flowers and Esbenshade 1993). The advantages of double inseminations have been described by Hofmo (1991), Crabo and Dial (1992) and Steverink et al. (1999).

Several alternatives to conventional AI have been used to reduce the number of spermatozoa necessary for fertilization. In *in-vitro* fertilization (IVF) programmes 20–1000 sperm cells are sufficient to fertilize an oocyte (Rath et al. 1997, 1999) and recently single sperm injection into the ooplasm of mature oocytes was successfully used to produce piglets (Kim and Shim 2000; Kolbe and Holtz 2000; Probst et al. 2002). These techniques are useful tools for special applications for example in nucleus herds and in research but not for the commercial swine production.

Physiological aspects of sperm transportation

For the development of new AI strategies, it is important to understand the fate of the sperm cells on their way to the site of fertilization. After insemination, spermatozoa migrate through the uterine body, the uterine horns and the utero-tubal junction (UTJ) into the oviduct. In the ampulla of the oviduct, sperm cells interact with the oocytes and, after recognition, penetrate the zona pellucida and fertilize the oocyte. Sperm transport is a complex process, involving stimulation of the sow through the mating or insemination process itself, composition of the ejaculate or inseminate, activity and secretions of the female genital tract, hormones and immunogenic factors (Bower 1974; Einarsson 1980; Viring 1981; Claus et al. 1989; Drobnis and Overstreet 1992). One critical factor is the direction of the myometrial waves that transport the inseminate towards the UTJ (Viring et al. 1980), which depends on the status of the oestrous cycle (Zerobin 1968). Intensive contractility of the uterus directly after mating is responsible for rapid sperm transport to the oviduct (Bower 1974). However, this first wave does not contain a capacitated sperm population (Hawk 1983). The viability of these sperm cells is low, because their membranes are weak and they are pushed through the oviduct and out into the abdominal cavity (Overstreet and Cooper 1978; Overstreet 1983).

In the second wave, sperm cells are moved more slowly through the uterus. On their way they are surrounded by substances in the seminal plasma which prevent sperm cells from undergoing capacitation too early (Yanagimachi 1994; Hunter et al. 1998). Once the

sperm have reached the UTJ, these factors are no longer present and capacitation begins. At the UTJ and in the distal part of the oviductal isthmus a sperm reservoir is built up. Here sperm cells are selectively prepared for their final migration to fertilize the oocytes in the oviduct (Viring et al. 1980; Hunter 1981, 1984; Yanagimachi 1994). The reservoir is important to maintain the viability and integrity of sperm for about 24–42 h (Hunter 1988). Mburu (1997) showed that boar sperm are located at specific regions of the UTJ and the distal oviduct. Only spermatozoa that had close contact with the ciliary epithelial cells had intact membranes and were classified as fertile. Similar observations were obtained *in vitro* when intact ciliary epithelial cells were able to bind spermatozoa (Suarez et al. 1991) and it seems that only non-capacitated sperm bind to the epithelial cells (Fazeli et al. 1999). A few spermatozoa are released and reach the oocytes, eventually guided by a temperature gradient (Hunter and Nichol 1986; Hunter 1995). After 2 days, the number of sperm cells at the UTJ decreases gradually (Polge 1978; Viring and Einarsson 1981).

Only a minor fraction of the inseminated spermatozoa reach the oviductal ampulla intact (Du Messnil Du Buisson and Dauzier 1955; Rigby 1964; Hunter 1973; Scott and Overstreet 1999). Additionally, the volume of the ejaculate/insemination dose is quickly reduced. Approximately 75 min after insemination, almost no seminal fluid/extender can be recovered (Lovell and Getty 1968). A major reason for sperm loss is the back flow of sperm cells and seminal plasma/extender after mating or insemination. Approximately 25–30% of the inseminated sperm cells, and up to 70% of the volume, are excreted retrogradely (Hunter 1973; Viring and Einarsson 1981; Steverink et al. 1998). In the pig, backflow seems to be a physiological event; but only affected the fertilization rate when the total number of sperm was $< 1 \times 10^9$ 80 ml (Steverink et al. 1998). If the backflow occurred not at insemination but 0.5–2.5 h later, no effects were observed on the establishment of a sufficient sperm reservoir at the UTJ. All trials to prevent backflow with a tamponade in the cervix failed to increase the sperm number in the upper uterus and the oviduct (Pursel 1982).

Further sperm losses are caused by adhesion of sperm cells to ciliary epithelial cells of the endometrium and by migration into uterine glands. Additionally, polymorphonuclear leucocytes diminish the sperm population within 30 min (Lovell and Getty 1968; Pursel et al. 1978; Hadjisavas et al. 1994; Scott and Overstreet 1999).

New AI strategies

Based on the physiological regulation of sperm transport to the oviduct, it may be advantageous to reduce sperm losses by depositing the sperm cells closer to the UTJ. The objectives of some basic experiments that were recently performed in gilts and sows were: (a) to determine the minimal number of sperm cells that are required for surgical insemination into the tip of the uterine horn (Krüger et al. 1999; Krüger and Rath 2000) and (b) to develop an insemination protocol that will allow non-surgical low dose insemination with sex selected or unselected semen (Wolken et al. 2002; Martinez et al. 2000; Roca et al. 2002; Vazquez et al. 2002).

Hancock (1959) and Hancock and Hovell (1961) reported the first intra-uterine inseminations in sows using 100×10^6 to 10×10^9 spermatozoa diluted in 20 or 120 ml of extender. Interestingly the fertility was better when only 20 ml was used for insemination. No difference between groups was seen in the pregnancy rate, but litter sizes differed significantly. Polge et al. (1970) obtained high fertilization rates in pigs when about 1×10^7 frozen thawed sperm in 0.5 ml extender were directly inseminated into the oviduct 6 h prior to the estimated time of ovulation. Similar results were achieved by Schoenbeck and Didion (1995) under farm conditions when they surgically inseminated frozen thawed semen into the oviduct. Piglets have also been produced after insemination of about 200 000 sexed sperm directly into the oviduct (Johnson 1991). The disadvantage of a protocol based on insemination directly into the oviduct is that it will never be possible to do this non-surgically.

Our initial experiments involved surgical insemination into the tips of the uterine horns (Krüger et al. 1999; Krüger and Rath 2000). Prepuberal gilts, hormonally stimulated with equine Chorionic Gonadotropine (eCG) and human Chorionic Gonadotropine (hCG), were inseminated with 1×10^9, 1×10^8, 1×10^7 5×10^6 and 1×10^6 sperm per uterine horn at 38 h or 32 h after hCG treatment or at the time of ovulation. Pregnancy rates, farrowing rates and litter size did not differ significantly between these groups if more than 1×10^6 sperm cells were used in 0.5 ml of extender. In this experiment, gilts were usually not allowed to go to term, but were slaughtered 48h after insemination and embryos were collected and evaluated with respect to integrity and then cultured *in vitro* for 5 days to determine their developmental potential. The reason for this final part of the experiment was that after low dose insemination, it is possible that defective spermatozoa have fertilized the oocytes (Saacke et al. 1998), because the normal competition between sperm cells would be diminished at such low sperm concentrations. However, no indications were found for this as long as the inseminated sperm number was higher than 1×10^6 sperm. These results may differ among boars.

Again, at the lowest sperm dosage (1×10^6), significantly less oocytes were fertilized than in the other groups. Additionally, embryos derived from the lowest concentration group had less developmental competence, as their cell cycles were prolonged from the second cell cycle onwards and none of the embryos in this group developed to hatched blastocyst stage. This may indicate that spermatozoa with non-compensatable defects participated in fertilization.

In the experiment with the stimulated gilts, the litter sizes of all groups were lower than one would expect from normal insemination, but was not dependent on sperm dosage nor on the time of insemination. In order to exclude hormonal and age effects, the experiment was repeated with sows after weaning and 1×10^9, 1×10^8 and 1×10^7 spermatozoa were inseminated per horn surgically. Additionally two groups were inseminated once intra-cervically (conventional AI) with 1×10^9 or 3×10^9 sperm. The results from all groups did not differ significantly and litter sizes were similar to what could be expected after conventional AI. As long as more than

1×10^6 spermatozoa are deposited close to the UTJ (at least under research conditions), normal pregnancy rates and litter sizes can be expected.

In a subsequent experiment we tested whether insemination into the distal part of the uterine horn gave similar results. Semen ($1 \times 10^7 - 1 \times 10^8$ spermatozoa in 0.5 ml, 10 and 50 ml Androhep) was deposited about 10 cm from the *bifurcatio uteri* into each horn. After 48 h embryos were flushed from the genital tract after slaughter. No differences were found for cleavage rates resulting from the different sperm concentrations and no differences were seen with respect to insemination volume (Wolken 2001).

We also compared insemination into the distal part of one horn vs both horns vs uterine body using similar sperm concentrations (Wolken 2001). No differences in fertilization rates were observed among these experimental groups. Cleavage rates were not affected by the site of semen deposition, i.e. there was no difference between ipsi- and contra-lateral horns. It was concluded that independent from the site of insemination, oocytes from both ovaries could be fertilized after insemination into the distal part of the uterus. How the spermatozoa fertilize the oocytes of the contra-lateral side is a subject for further studies.

For non-surgical AI into the uterus, Martinez et al. (2000) used a modified flexible-fibre endoscope that they pushed forward to the tip of the uterine horn. Pregnancy rates ranged from 87 to 89% and the litter size was raised from 9.4 to 9.8 piglets. Vazquez et al. (1999) had shown before that in more than 76% of sows no handling problems occurred when semen was deposited with the flexible catheter into the tip of the uterus horn and 20×10^6 sperm cells diluted in 5 ml of extender were sufficient to produce pregnancies in all inseminated sows. Roca et al. (2002) used the intra-uterine insemination technique to inseminate with frozen/thawed semen. No differences in pregnancy rate and litter size were found between thawed semen and fresh semen. Therefore, intra-uterine insemination technique will be an ideal technology to broaden the use of frozen/thawed semen in pigs. Initial trials have also been performed with flow cytometrically selected spermatozoa (bulk sort) and have indicated that flow cytometry can yield sperm which then produce piglets of desired properties (Vazquez et al. 2002).

Another approach has been described by Wolken et al. (2002), after they found that it is not necessary to inseminate deep into the uterine horn. Instead of depositing the semen into the tip of the uterine horn, spermatozoa were deposited non-surgically into the distal uterine horn approximately 30 cm distant from the *bifurcatio uteri.*

In a field trial, approximately 100 sows were inseminated after weaning with a specially designed insemination device (Minitüb, Tiefenbach, Germany). The final concentration of spermatozoa was 5×10^8/20 ml, 1×10^8/20 ml or 1×10^8/10 ml and insemination was performed only once per oestrus. A control group was inseminated following the conventional AI protocol with 3×10^9 sperm/100 ml. Sows were slaughtered 28–35 days after insemination. Based on the foetuses recovered, pregnancy rates did not differ significantly between groups, but were somewhat higher when the insemination volume was 20 ml. Additionally, no differences were found in comparison with the conventional AI control group. The number of foetuses did not differ among the experimental groups and were similar to the litter size from the previous pregnancy. The author concluded that distal insemination can successfully be performed, although it seems that the minimal sperm concentration deposited into the distal horn needs to be higher than for insemination into the tip of the uterine horn. Distal insemination is easier to perform and may reduce the risk of injury and infection of the uterine horns.

Conclusion

In conclusion, deep intra-uterine insemination in pigs is a possibility to optimize the use of ejaculates and to introduce new sperm based technologies into pig reproduction. It will also be advantageous for frozen/thawed semen and as only a low volume is required, freezing protocols might be modified for low dose insemination. The first instruments for intra-uterine insemination are now available and will allow insemination with less sperm with similar reproduction success as with conventional insemination techniques. It is important to mention that in all experiments boars were selected for the highest sperm quality. Boar differences become critical as the number of sperm cells is reduced. Only those boars that fulfil requirements for semen quality should be used for AI in future.

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(54) **JACKETED VESSEL FOR HOLDING SEMEN FOR SEX BIASING MAMMALS THROUGH ARTIFICIAL INSEMINATION AND SYSTEMS AND METHODS FOR ENHANCING THE PROBABILITY OF SEX BIASING USING THE SAME**

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(52) **U.S. Cl.** **435/2**; 435/284.1

(57) **ABSTRACT**

A jacketed container for holding a semen sample from a mammalian donor at a predetermined temperature for a predetermined period of time prior to incubation to enhance the probability of obtaining offspring of a selected sex through artificial insemination is disclosed. In one embodiment, the semen sample is collected and insulated by a high-heat capacity material that has been preconditioned to a temperature between about 30° C. to about 40° C. before the semen is cooled to a temperature of about 12° C. In a second embodiment, the semen sample is collected and insulated by a high-heat capacity material that has been preconditioned to a temperature between about 4° C. to about 20° C. before the semen is cooled to a temperature of about 12° C.

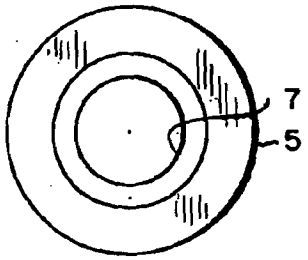

FIG. 3

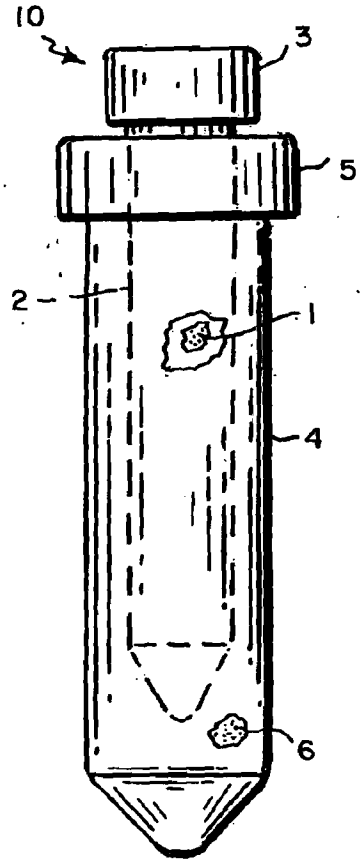

FIG. 1

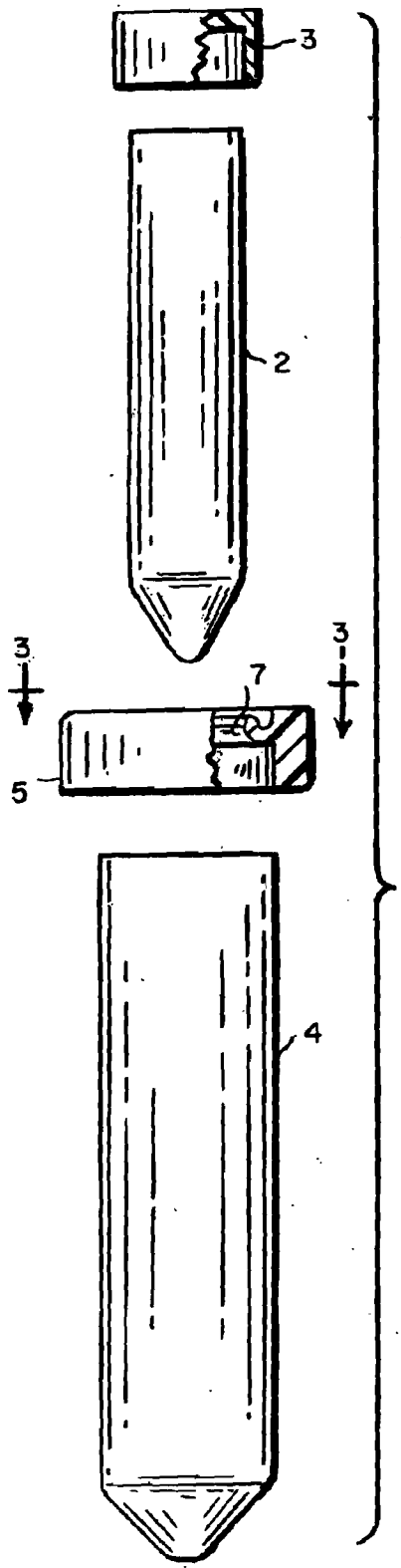

FIG. 2

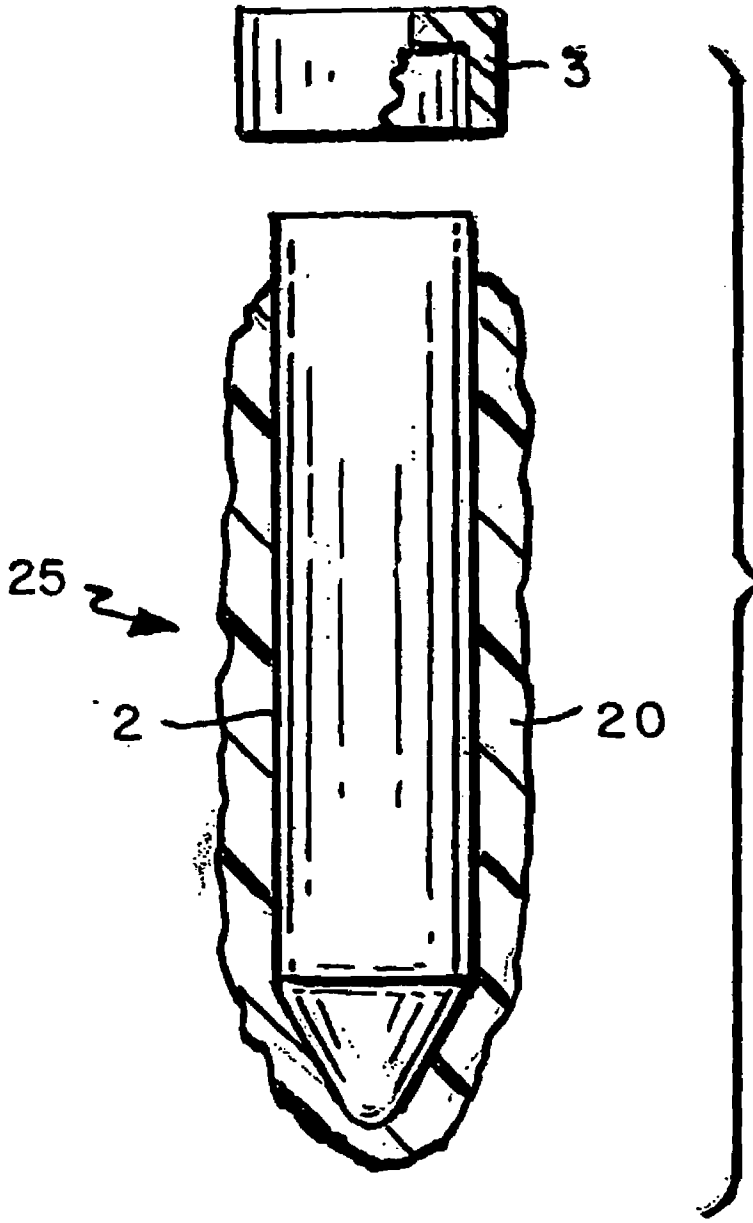

FIG. 4

JACKETED VESSEL FOR HOLDING SEMEN FOR SEX BIASING MAMMALS THROUGH ARTIFICIAL INSEMINATION AND SYSTEMS AND METHODS FOR ENHANCING THE PROBABILITY OF SEX BIASING USING THE SAME

RELATIONSHIP TO PREVIOUS APPLICATIONS

[0001] The present invention claims priority of provisional U.S. Patent Application No. 60/641,062 entitled "Method for sex biasing of artificial insemination" that was filed on Dec. 30, 2004.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] This invention relates to devices, systems, and methods for enhancing the probability of obtaining offspring of a selected sex. More particularly, this invention relates to devices, systems, and methods for collecting spermatozoa prior to artificial insemination to enhance the probability of obtaining offspring of a selected sex.

[0004] 2. Background of the Related Art

[0005] Agricultural sexing is considered by many to be one of if not the most sought after technology of the current millennium. Indeed, farmers and others practicing animal husbandry have long recognized the desirability of enhancing the probability of obtaining offspring of a selected sex. For example, in the dairy industry, there is a greater demand for milk-producing heifers than for their male counterparts. Consequently, there is a need for methods and devices to artificially bias the sex of mammalian offspring with a high degree of certainty.

[0006] In mammals, the male gamete, or spermatozoan controls the sex of offspring. Each spermatozoan contains either an X-type or a Y-type sex-determining chromosome. An X-chromosome spermatozoan creates female offspring after fertilization with an oocyte, whereas a Y-chromosome spermatozoan creates male offspring after fertilization. Methods have been proposed for increasing the percentage of X-chromosome bearing sperm cells or Y-chromosome bearing sperm cells to achieve a greater probability of achieving female or male offspring, respectively.

[0007] For example, previous methods have included methods based upon density sedimentation. See, for example, Brandriff, B. F. et al. "Sex Chromosome Ratios Determined by Karyotypic Analysis in Albumin-Isolated Human Sperm," Fertil. Steril., 46, pp. 678-685 (1986), which is incorporated herein by reference.

[0008] U.S. Pat. No. 3,687,806 to Van Den Bovenkamp discloses an immunological method for controlling the sex of mammalian offspring using antibodies that react with either X-bearing sperm or Y-bearing sperm, which utilizes an agglutination step to separate bound antibodies from unaffected antibodies.

[0009] U.S. Pat. No. 4,083,957 to Lang discloses a method of altering the sex ratio in animal (including human) offspring by separating the population of spermatozoa into fractions that differ by the sex-linked electrical charge resident thereon. The separation is carried out by bringing the spermatozoa into close association with an electrostatic charge-bearing material having a charge the sign of which is opposite to the sign of a chosen portion of the spermatozoa, that portion that carries the sex-determining character of the unwanted sex, so as to attract and thereby to permit that portion to be isolated, or put to a disadvantage in the fertilization of ova. Concern is expressed with the selection of the charge-bearing material, the adjustment of the pH and particle size thereof, and the control of the surrounding medium in relation to its influence on the charge characteristics of both the charge-bearing material and the spermatozoa. Lang teaches that spermatozoa having male or female sex-bearing genetic material also have differing electrostatic charges—normally negative for male and positive for female—and, further, uses this teaching for separating male and female spermatozoa with charge-bearing materials.

[0010] U.S. Pat. No. 4,191,749 to Bryant discloses a method for increasing the percentage of mammalian offspring of either sex by use of a male-specific antibody coupled to a solid-phase immunoabsorbant material to selectively bind male-determining spermatozoa, while the female-determining spermatozoa remain unbound in a supernatant.

[0011] U.S. Pat. No. 5,021,244 to Spaulding discloses a method for sorting living cells based upon DNA capacity, particularly sperm populations to produce subpopulations enriched in X-sperm or Y-sperm by means of sex-associated membrane proteins and antibodies specific for such proteins.

[0012] U.S. Pat. No. 5,514,537 to Chandler discloses a method and apparatus for the mechanical sorting of mammalian spermatozoa by sex-type into a fraction enriched in X-chromosome-bearing spermatozoa and into a fraction enriched in Y-chromosome-bearing spermatozoa. Because of their different DNA capacity, Y-chromosome spermatozoa are on average slightly smaller than X-chromosome spermatozoa. According to Chandler, a column can be packed with two sizes of beads. The size of the smaller beads is chosen such that, on average, Y-chromosome spermatozoa will readily fit into the interstices between the smaller beads, whereas X-chromosome spermatozoa, on average, will not readily fit into those interstices. The size of the larger beads is chosen such that the larger beads will readily fit into the interstices between the larger beads and the smaller beads will pass through the interstices. As a result, a liquid sample containing sperm is run through a column so that the liquid first encounters the larger beads, and, subsequently, encounters the smaller beads. The beads act as a sieve, creating a fraction in the larger beads enriched in X-chromosome spermatozoa, and a fraction in the smaller beads enriched in Y-chromosome spermatozoa.

[0013] However, these prior art methods often result in insufficient separation of X-sperm and Y-sperm and often damage the sperm, thereby reducing its motility and fertility success rate. As a result, in commonly owned and assigned U.S. Pat. Nos. 6,153,373 and 6,489,092, improved methods for sex determination of mammalian offspring are provided using antibodies coupled to magnetic particles for separation of spermatozoa. These methods use magnetic separation to provide gentle separation of populations of spermatozoa.

[0014] Lechniak, et al. in *Reprod Dom Anim* 38, 224-227 (2003), which is incorporated herein by reference, describe a study to determine whether or not sperm pre-incubation prior to fertilization in vitro (IVF) influences the rate of fertilization, embryo development, and/or the sex ratio

among blastocysts. In the study, oocyte-cumulus-complexes (OCC) were aspirated from follicles of slaughterhouse ovaries; collected in Hepes-buffered Ham's F-10; and matured in maturation medium under silicone oil for 24 hours at 39 degrees Centigrade (° C.). Frozen-thawed sperm cells were utilized. After swim-up, the motile fraction of sperm was incubated in Sperm-Talp (no heparin included) at 39° C. for 0, 6 and 24 hours. Sperm count was carried out and sperm motility was evaluated. The number of motile sperm cells was kept similar in each experimental group. The motile spermatozoa decreased with time. It was reported by the authors that, when comparisons between groups were made and the actual sex ratios taken into consideration, there were significantly more female-hatched blastocysts among the 24-hour group than among those of either the 0- or 6-hour pre-incubation groups. Unfortunately, IVF is not a practical procedure for fertilization of large herds.

[0015] Therefore, it would be desirable to provide novelty devices, systems, and methods of collecting semen to enhance the probability of sex biasing in artificial insemination.

SUMMARY OF THE INVENTION

[0016] The present invention discloses devices, systems, and methods for collecting and preparing a specimen of semen, i.e., a semen ejaculate, to increase the relative number of offspring of a preferred sex in mammals using artificial insemination (AI). For example, a specimen of semen ejaculate can be collected from a mammalian male donor in a jacketed collection tube having a predetermined collection temperature. After collection, the specimen in the jacketed collection tube can be cooled to a predetermined temperature, typically in the range of about 4° C. to about 20° C.; and the specimen can be incubated at that predetermined temperature for a predetermined period of time, typically in the range of from about 2 hours to about 24 hours. After incubation for the predetermined period of time, the specimen is processed into straws, which are used for artificial insemination in a corresponding female mammal using conventional procedures. Preferably, the straws can be frozen before conventional AI use. By treating the semen ejaculate as described, it has been found that a significant bias can be obtained in producing mammalian offspring of a preferred sex by AI. More important, by controlling the temperature of the semen ejaculate early on in the collection process, the rate of success is significantly improved.

[0017] Indeed, in a preferred embodiment of the present application, at time zero, mammalian semen ejaculate can be collected in a collection tube and encased in a jacketed container containing a high-heat capacity material. Preferably, the jacketed container contains a material that retains heat and dissipates heat very slowly. The high-heat capacity material can be preconditioned to about 32° C. or, alternatively, to about 12° C.

[0018] More specifically, in one embodiment, the present invention provides a system for collecting and handling a specimen of semen ejaculate useful for artificial insemination to increase the conception of mammalian offspring of a preferred sex, wherein the system comprises an inner collection container for collecting and holding a semen ejaculate from a donor; and an outer, temperature control container that contains a high-heat capacity material for maintaining the specimen of semen ejaculate at a predetermined temperature for a predetermined period of time. Preferably, the high-heat capacity material and outer, temperature control container are preconditioned to a predetermined temperature in the range of about 4° C. to about 20° C. More preferably, the high-heat capacity material and outer, temperature control container are preconditioned to a predetermined temperature in the range of about 30° C. to about 40° C.

[0019] In one aspect of this embodiment of the invention, the jacketed inner collection container remains in the high-heat capacity material at one temperature range or the other for a predetermined period of time before the jacketed contained is immersed in a water bath. Preferably, the predetermined period of time before the jacketed container is immersed in a cooling water bath is less than about five minutes. More preferably, the predetermined period of time before the jacketed container is immersed in a cooling water bath is about one minute.

[0020] In another embodiment, the present invention provides an apparatus for collecting and handling a specimen of semen ejaculate useful for artificial insemination to increase the conception of mammalian offspring of a preferred sex, wherein the apparatus comprises a collection container for collecting and holding a semen ejaculate from a donor. Preferably, the container includes an outer coating that comprises a high-heat capacity material for maintaining the semen ejaculate specimen at a predetermined temperature for at least a predetermined period of time. In one aspect of this embodiment, the high-heat capacity material comprises a non-toxic refrigerant gel.

[0021] Preferably, the high-heat capacity material is preconditioned to a predetermined temperature in the range of about 4° C. to about 20° C. More preferably, the high-heat capacity material is preconditioned to a predetermined temperature in the range of about 30° C. to about 40° C. In one aspect of this embodiment of the invention, the jacketed inner collection container remains in the high-heat capacity material at one temperature range or the other for a predetermined period of time before the coated contained is immersed in a cooling water bath. Preferably, the predetermined period of time before the coated container is immersed in a water bath is less than about five minutes. More preferably, the predetermined period of time before the coated container is immersed in a water bath is about one minute.

[0022] In yet another embodiment, the present invention provides a method of collecting and handling a specimen of semen useful for artificial insemination to increase the conception of mammalian offspring of a preferred sex, the method comprising the steps of preconditioning a high-heat capacity material; collecting and handling a specimen of semen useful for artificial insemination; encasing the specimen of semen in the high-heat capacity material; and immersing the encased specimen of semen in a temperature controlled device to cool the specimen of semen in a controlled manner.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] The invention will be better understood by reference to the following more detailed description and accompanying drawings where like reference numbers refer to like parts:

[0024] **FIG. 1** is an illustrative embodiment of a jacketed assembly in accordance with the present invention;

[0025] **FIG. 2** is an exploded view of the jacketed assembly in **FIG. 1** in accordance with the present invention;

[0026] **FIG. 3** is a plan view of a jacketed assembly in accordance with the present invention; and

[0027] **FIG. 4** is an illustrative embodiment of a coated assembly in accordance with the present invention.

DETAILED DESCRIPTION OF THE INVENTION INCLUDING PREFERRED EMBODIMENTS

[0028] The present invention provides devices, systems, and methods for collecting and incubating competent, viable sperm to fertilize mammalian eggs, e.g., inter alia, eggs in fertile cows, using standard AI techniques currently employed on farms. As noted above, prior methods to bias offspring production often compromise sperm integrity, e.g., sperm motility and/or fertilization ability, so that fertilization utilizing such prior art treated sperm requires complicated techniques such as in vitro fertilization (IVF) or ultrasounding of cows during heat to determine side of ovulation, coupled with introduction of a low sperm dose by high uterine horn insemination into the horn attached to the ovary from which the egg is released. It is impractical, however, to use these methods on dairy farms with working dairy herds. The method of the present invention can be utilized for sperm from a variety of mammalian species, including various livestock, such as cattle and sheep, as well as dogs, cats, horses, swine, and other species. The process also is applicable to humans.

[0029] We will now describe a first embodiment of the present invention. Referring to **FIGS. 1-3**, there are shown a device **4** and system **10** for controlling the temperature of a collected semen ejaculate **1** during a crucial portion of the collection process. In commercial AI applications, semen ejaculate **1** is collected from a male donor, e.g., a proven artificial insemination bull, i.e., an elite bull. Among the methods of collecting semen ejaculate include artificial vaginas, an electro-ejaculator, and the like. With an artificial vagina, typically, the elite bull is induced to complete a false mounting. Subsequently, the semen ejaculate **1** is collected in a collection container **2**, i.e., a collection tube **2**, that is disposed at the end of the artificial vagina.

[0030] Collection tubes **2** can be disposable and, further, can be made of, for example, plastic, resin, glass, polystyrene, polyethylene terephthalate (PET), and the like. PET centrifuge tubes manufactured by Corning of Corning, N.Y. are especially suitable for such use. The size, i.e., volume, of the collection tube **2** can be adjusted for the particular mammal species and the particular donor. Thus, a range of collection tube sizes ranging from about 1 ml to about 25 ml is generally suitable for the present invention. Typically, for a bull, a collection tube **2** in the range between about 10 ml to about 20 ml and, preferably a 15 ml collection tube **2** is suitable.

[0031] The assembly **10**, further, comprises an outer, temperature control container **4** that is structured and arranged to encompass and/or insulate, i.e., jacket, the inner, collection tube **2**. The size, i.e., volume, of the temperature control container **4**, or jacket **4**, can be selected to be compatible with the size of the collection tube **2**. For example, typically, for a 15 ml collection tube **2**, a 50 ml jacket **4** can be used. Temperature control containers **4** should be disposable and can be made of, for example, plastic, resin, glass, polystyrene, polyethylene terephthalate (PET), and the like. PET centrifuge tubes manufactured by Corning of Corning, N.Y. are especially suitable for such use.

[0032] Preferably, the outer, temperature control container **4**, or jacket **4**, is larger than the collection tube **2** and contains a high-heat capacity material **6** to control the temperature of the semen ejaculate **1** during a critical portion of the sex biasing process. Any suitable high-heat capacity material **6** can be used in conjunction with the jacket **4** as long as it has adequate heat transfer properties to maintain the semen ejaculate **1** at the desired temperature for a predetermined period of time. In a preferred embodiment, the high-heat capacity material **6** is a viscous gel such as non-toxic refrigerant, phase change gels such as U-TEK®+30° F. or U-TEK®−10° F. or Polar Pack products. Starch-based gels are suitable for this use but they are less preferred because they liquefy with time, which can create a mess. Various materials **6** such as insulating materials also can be used.

[0033] Immediately after collection of the semen ejaculate **1** at the artificial vagina but prior to incubation, research performed by the inventors has demonstrated that the semen ejaculate **1** enters a crucial period of the collection process, i.e., a window of opportunity, for the purpose of AI sex biasing. Specifically, the inventors have demonstrated that controlling the temperature of the semen ejaculate **1** immediately after collection and prior to incubation impacts sex biasing. More particularly, the inventors have demonstrated that controlling the temperature of the semen ejaculate **1** during the first minute or two after collection and prior to incubation impacts sex biasing.

[0034] In a preferred embodiment, the semen ejaculate **1** is collected in a collection container **2**, or collection tube **2** from the end of an artificial vagina (not shown). The collection tube **2** is capped, e.g., using a cap **3**, to avoid loss or spillage and, then, quickly encased or immersed in a temperature control container **4** that, preferably, is filled with a high-heat capacity material **6**, e.g., a freezer pack gel, that has been preconditioned to a desired temperature. In this embodiment, before collection of the semen ejaculate **1**, the temperature control container **4** and the high-heat capacity material **6** are preconditioned, i.e., preheated, to a temperature in the range between about 30° C. and about 40° C., preferably in the range between about 32° C. and about 35° C., e.g., in a water bath (not shown). As a result, when the collection tube **2** containing the freshly collected semen ejaculate **1** is introduced into the high-heat capacity material **6** in the jacket **4**, the high-heat capacity material **6** maintains the semen ejaculate **1** at or near preconditioning temperature, which is about the same as the temperature of the semen ejaculate **1** upon ejaculation and collection from the donor.

[0035] In one aspect of the present embodiment, the collection tube **2** is inserted through an opening (not shown) in the cap **5** of the temperature control container **4**. The opening can be a circular opening, where a substantially circular portion has been removed from the middle portion **7** of the cap **5** or, alternatively, the opening can comprise an "X" pattern that has been cut, e.g., along the diameter of the

middle portion **7** of the cap **5**. Optionally, to minimize loss by seepage of the high-heat capacity material **6**, a gasket or O-ring (not shown) can be included to seal any gaps between the opening in the cap **7** and the outer surface of the collection tube **2**. Furthermore, spacers and/or ribs (not shown) can be optionally included in the temperature control container **4** to center the collection tube **2** within the temperature control container **4** to distribute the temperature more uniformly. Such seals, spacers, and ribs are well known to those skilled in the art and will not be described further.

[0036] The jacketed collection tube **2**, i.e., the assembly **10**, is then promptly placed, or immersed, into another temperature-controlled device, e.g., a circulating water bath (not shown), and cooled, as necessary, to a predetermined temperature. Preferably, the temperature of the cooling water bath is in a range between about 4° C. and about 20° C., or, more preferably, in the range between about 6° C. and about 17° C., or, most preferably about 12° C.

[0037] Preferably, the collection tube **2** is jacketed in the outer, temperature control container **4**, and the entire assembly **10** is placed in a circulating water bath within about 5 minutes of collection of the semen ejaculate **1**. More preferably, the jacketed collection tube **2**, i.e., the assembly **10**, is placed in a circulating water bath within about 3 minutes of collection. Most preferably, the jacketed collection tube **2**, i.e., the assembly **10**, is placed in a circulating water bath within about 1 minute of collection.

[0038] The collected semen ejaculate **1** is then incubated in the cold-water bath, e.g., at about 12° C., for another predetermined period of time. Typically, the predetermined period of time is from about 2 hours to about 24 hours. Preferably, the predetermined period of time is from about 2 hours to about 12 hours. More preferably, the predetermined period of time is from about 2 to about 8 hours. Most preferably, the predetermined period of time is from about 4 to about 6 hours.

[0039] After incubation, the cooled semen ejaculate **1** is extended, e.g., using phosphate buffered saline ("PBS"), to the desired volume and straws are prepared according to conventional procedures. The straws can be frozen and stored prior to use for artificial insemination. Typically, the straws are thawed and semen from a straw deposited in the uterus just beyond the cervix.

[0040] In another preferred embodiment, the semen ejaculate **1** is collected in a collection container **2**, or collection tube **2**, from the end of an artificial vagina (not shown). The collection tube **2** is capped, e.g., using a cap **3** to avoid loss or spillage, and, then, encased or immersed in a temperature control container **4** that, preferably, is filled with a high-heat capacity material **6**, e.g., a freezer pack gel, that has been preconditioned to a desired temperature. In this embodiment, before collection of the semen ejaculate **1**, the temperature control container **4** containing the high-heat capacity material **6** is preconditioned, i.e. pre-cooled, to a temperature in a range between about 4° C. and about 20° C., preferably, in the range of about 6° C. to about 17° C., e.g., in a cool water bath (not shown). As a result, when the collection tube **2** containing the freshly collected semen ejaculate **1** is introduced into the high-heat capacity material **6** in the jacket **4**, the high-heat capacity material **6** immediately begins to cool the semen ejaculate **1**.

[0041] In one aspect of this preferred embodiment, the collection tube **2** is inserted through an opening (not shown) in the cap **5** of the temperature control container **4**. The opening can be a circular opening, where a substantially circular portion has been removed from the middle portion **7** of the cap **5** or, alternatively, the opening can comprise an "X" pattern that has been cut, e.g., along the diameter of the middle portion **7** of the cap **5**. Optionally, to minimize loss by seepage of the high-heat capacity material **6**, a gasket or O-ring (not shown) can be included to seal any gaps between the opening in the cap **7** and the outer surface of the collection tube **2**. Furthermore, spacers and/or ribs (not shown) can be optionally included in the temperature control container **4** to center the collection tube **2** within the temperature control container **4** to distribute the temperature more uniformly. Such seals, spacers, and ribs are well known to those skilled in the art and will not be described further.

[0042] The jacketed collection tube **2**, i.e., the assembly **10**, is then promptly placed, or immersed, into another temperature-controlled device, e.g., a circulating water bath (not shown), and cooled, as necessary, to a predetermined temperature.

[0043] Preferably, the temperature of the cooling water bath is in a range between about 4° C. and about 20° C., or, more preferably, in the range between about 6° C. and about 17° C., or, most preferably about 12° C. Preferably, the collection tube **2** is jacketed in the outer, temperature control container **4**, and the entire assembly **10** is placed in a circulating water bath within about 5 minutes of collection of the semen ejaculate **1**. More preferably, the jacketed collection tube **2**, i.e., the assembly **10**, is placed in a circulating water bath within about 3 minutes of collection. Most preferably, the jacketed collection tube **2**, i.e., the assembly **10**, is placed in a circulating water bath within about 1 minute of collection.

[0044] The collected semen ejaculate **1** is then incubated in the cold-water bath, e.g., at about 12° C., for another predetermined period of time. Typically, the predetermined period of time is from about 2 hours to about 24 hours. Preferably, the predetermined period of time is from about 2 hours to about 12 hours. More preferably, the predetermined period of time is from about 2 to about 8 hours.

[0045] Most preferably, the predetermined period of time is from about 4 to about 6 hours.

[0046] After incubation, the cooled semen ejaculate **1** is extended, e.g., PBS, to the desired volume and straws are prepared according to conventional procedures. The straws can be frozen and stored prior to use for artificial insemination. Typically, the straws are thawed and semen from a straw deposited in the uterus just beyond the cervix.

[0047] Although, up to this point, the invention has been described by introducing a collection tube **2** into a container **4** containing a high-heat capacity material **6** after collection of the semen ejaculate **1**, in a second embodiment, referring to **FIG. 54**, the outer surface of the collection tube **2** can, instead, be coated with a high-heat capacity material **20**, e.g. a viscous gel layer, that has sufficient stability to be handled without losing the heat transfer properties of the gel layer **20** and sufficient viscosity to remain in physical communication with the collection tube **2** when it is incubated in a cooling water bath as described above.

[0048] For example, a collection tube **2** can be immersed, i.e., dunked, in a high-heat capacity material **6** so that a viscous gel layer **20** adheres to the outer surface of the collection tube **2**. Immersion can occur before or after the semen ejaculate **1** is collected. Alternatively, a viscous gel layer **20** of high-heat capacity material **6** can be applied or spread, e.g., using a spatula, on the outer surface of the collection tube **2**.

[0049] By collecting semen ejaculate **1** using a jacketed assembly **10** or a coated assembly **25** to control and maintain the temperature of the semen **1** immediately after collection and prior to incubation, the spermatozoa of a mammal can be incubated and processed without a substantial loss of quality. Quality includes, but is not limited to: motility, progressive motility, grade of motility, acrosomal integrity, immediate and incubated post-thaw motility, and morphology. Consequently, the quality of the incubated spermatozoa using a jacket assembly **10** is at least about 50% of the unprocessed spermatozoa. Preferably, the functionality of the fractionated spermatozoa is at least about 60% of the unprocessed spermatozoa, at least about 70% of the unprocessed spermatozoa, at least about 80% of the unprocessed spermatozoa, or is at least about 90% of the unprocessed spermatozoa. More preferably, the quality of the fractionated spermatozoa is at least about 95% of the unprocessed spermatozoa, still more preferably is at least about 97% of the unprocessed spermatozoa, yet even more preferably is at least about 98% of the unprocessed spermatozoa, and most preferably is at least about 99% of the unprocessed spermatozoa. Thus, populations of incubated spermatozoa preferentially determinative of one sex having the foregoing levels of quality relative to unprocessed spermatozoa are provided.

[0050] The invention will be described further in the following example.

EXAMPLE 1

[0051] Semen ejaculate **1** was collected from proven artificial insemination bulls according to the following procedure.

[0052] 1. Ejaculate **1** was collected at ambient temperature into a modified collection assembly **10** comprising of a 15 ml collection tube **2** that has been immersed in a container **4** of freezer pack gel **6** that was preconditioned to a temperature of about 32° C. prior to use. Immediately before the ejaculate **1** was collected but after the completion of the required false mountings, the collection apparatus **2** was attached to the end of the artificial vagina and the ejaculate collected.

2. The insulated collection assembly **10** was transferred to a circulating water bath at 12° C. to begin the cooling incubation process.

3. Incubate for 6 hours at 12° C.

4. Prepare straws according to conventional procedures.

[0053] Conveniently, a jacketed collection tube **10** was made by filling a 50 ml conical tube **4** with freezer pack gel **6**, covering the top with a cap, e.g., a centrifuge tube cap, in which a cross-cut opening was made, and inserting into the gel **6** through the opening a 15 ml conical tube **2** into which the semen **1** was collected.

[0054] The collected and incubated semen was extended and frozen using conventional freezing procedures. Straws were prepared at about 20 million sperm cells/straw (calculated).

[0055] Cows and heifers in working dairy herds were inseminated with semen by artificial insemination (AI) with the incubated semen and with a control semen. The results of the AI are tabulated in Table 1.

TABLE 1

Straw	Female Feti	Male Feti	Total Feti	% Female
Sexed	92	53	145	63.4%
Control	62	63	125	49.6%

[0056] The invention has been described in detail including preferred embodiments thereof. However, modifications and improvements within the scope of this invention will occur to those skilled in the art. The above description is intended to be exemplary only. The scope of this invention is defined only by the following claims and their equivalents.

[0057] For example, although the invention has been described having an outer coating **20** or a temperature control device **4**, i.e., a jacket **4**, the invention is not to be construed as being limited thereto. Indeed, a covering, e.g., a ceramic or polymer covering, can be used in conjunction with the coating **20**.

What is claimed is:

1. A system for collecting and handling a specimen of semen useful for artificial insemination to increase the conception of mammalian offspring of a desired sex prior to incubation, the system comprising:

an inner collection container for collecting and holding a semen ejaculate from a donor; and

an outer, temperature control container, containing a material therein that maintains the specimen of semen at a predetermined temperature for at least a predetermined period of time prior to initiation of incubation.

2. The system as recited in claim 1, wherein the material is a high-heat capacity material.

3. The system as recited in claim 1, wherein the material is preconditioned to a predetermined temperature in the range of about 4° C. to about 20° C.

4. The system as recited in claim 3, wherein the predetermined temperature is in the range of about 6° C. to about 17° C.

5. The system as recited claim 4, wherein the predetermined temperature is about 12° C.

6. The system as recited in claim 1, wherein the material is preconditioned to a predetermined temperature in the range of about 30° C. to about 40° C.

7. The system as recited in claim 6, wherein the predetermined temperature is in the range of about 32° C. to about 35° C.

8. The system as recited claim 7, wherein the predetermined temperature is about 32° C.

9. The system as recited claim 1, wherein the predetermined period of time is less than about five minutes.

10. The system as recited claim 9, wherein the predetermined period of time is less than about three minutes.

11. The system as recited claim 10, wherein the predetermined period of time is less than about one minute.

12. A method for increasing the conception of mammalian offspring of a preferred sex, the method comprising:

preconditioning a high-heat capacity material;

collecting and handling a specimen of semen useful for artificial insemination;

encasing the specimen of semen in the high-heat capacity material; and

immersing the encased specimen of semen in a temperature controlled device to cool the specimen of semen in a controlled manner.

13. The method as recited in claim 12, wherein the preconditioning step includes preconditioning the high-heat capacity material to a temperature in the range of from about 30° C. to about 40° C.

14. The system as recited claim 13, wherein the preconditioning step includes preconditioning the high-heat capacity material to a temperature in the range of from about 32° C. to about 35° C.

15. The method as recited in claim 12, wherein the preconditioning step includes preconditioning the high-heat capacity material to a temperature in the range of from about 4° C. to about 20° C.

16. The method as recited in claim 15, wherein the preconditioning step includes preconditioning the high-heat capacity material to a temperature in the range of from about 6° C. to about 17° C.

17. The method as recited in claim 12, wherein the encasing step includes immersing the specimen of semen in a high-heat capacity material that is contained in a temperature control container.

18. The method as recited in claim 12, wherein the encasing step includes encasing the specimen of semen in a high-heat capacity viscous gel.

19. The method as recited in claim 12, wherein the immersion step includes:

placing a jacketed collection tube into the temperature controlled device, and

controlling the temperature of the temperature controlled device in the range of from about 4° C. to about 20° C.

20. The method as recited in claim 19, wherein the immersion step includes:

placing a jacketed collection tube into the temperature controlled device, and

controlling the temperature of the temperature controlled device in the range of from about 6° C. to about 17° C.

21. The method as recited in claim 20, wherein the immersion step includes:

placing a jacketed collection tube into the temperature controlled device, and

controlling the temperature of the temperature controlled device at about 12° C.

22. The method as recited in claim 12, wherein the immersion step is performed for about 2 to about 24 hours to cool the specimen of semen to about 12° C.

23. An apparatus for collecting and handling a specimen of semen useful for artificial insemination to increase the conception of mammalian offspring of a preferred sex, the apparatus comprising:

a collection container for collecting a semen ejaculate from a donor, the container having an outer coating, wherein

the outer coating comprises a material for maintaining the specimen at a predetermined temperature for at least a predetermined period of time.

24. The apparatus as recited in claim 23, wherein the material is a high-heat capacity material.

25. The apparatus as recited in claim 24, wherein the material comprises a non-toxic refrigerant gel.

26. The apparatus as recited in claim 23, wherein the material is preconditioned to a predetermined temperature in the range of about 4° C. to about 20° C.

27. The apparatus as recited in claim 26, wherein the predetermined temperature is in the range of about 6° C. to about 17° C.

28. The apparatus as recited in claim 27, wherein the predetermined temperature is about 12° C.

29. The apparatus as recited in claim 23, wherein the material is preconditioned to a predetermined temperature in the range of about 30° C. to about 40° C.

30. The apparatus as recited in claim 29, wherein the predetermined temperature is in the range of about 32° C. to about 35° C.

31. The apparatus as recited in claim 30, wherein the predetermined temperature is about 32° C.

32. The apparatus as recited in claim 23, wherein the predetermined period of time is less than about five minutes.

32. The apparatus as recited in claim 31, wherein the predetermined period of time is less than about three minutes.

33. The apparatus as recited in claim 32, wherein the predetermined period of time is less than about one minute.

* * * * *

Exhibit B

MOLECULAR REPRODUCTION AND DEVELOPMENT 52:50–56 (1999)

Improved Flow Cytometric Sorting of X- and Y-Chromosome Bearing Sperm: Substantial Increase in Yield of Sexed Semen

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ABSTRACT The yield of flow cytometric sorted X- and Y-chromosome-bearing sperm in a given time period is an important factor in the strategies used for fertilization and the production of sex-preselected offspring. This yield is dependent on the efficiency with which the modified flow cytometer/cell sorter analyzes the DNA of spermatozoa. The efficiency is directly related to the number of sperm with the correct orientation during DNA analysis. Currently, the efficiency of flow cytometric sperm sorting is low since orientation of the sperm head to laser excitation is rate limiting. To overcome this problem, a new nozzle was designed to enhance sperm orientation and tested under flow cytometric sorting conditions. The degree of orientation improvement was determined with different sample rates using viable sperm and dead sperm of several different species. There was at minimum, a two-fold increase in the proportion of oriented sperm when comparing the new nozzle with the currently used modified flow cytometer/cell sorter employing a beveled needle. More than 60% of intact bull sperm and boar sperm were correctly oriented compared with 25% to 30% using the beveled needle system. A unique characteristic of the novel nozzle was that the proportion of oriented sperm was independent of sample rate and of sperm motility. The accuracy of DNA measurement together with high purity sorting was tested using the novel nozzle. The novel nozzle was unique in that accuracy of measurement and sorting performance were not diminished. Using the new nozzle, samples of 88% purity of sorted X-sperm and Y-sperm were obtained for viable bull and boar sperm. The yield of flow cytometric sorted X- and Y-chromosome-bearing sperm using the novel nozzle was, on average, twice that obtained by using the beveled needle system in conjunction with a standard equipment nozzle for orientation. *Mol. Reprod. Dev. 52:50–56, 1999.* Published 1999 Wiley-Liss, Inc.

Key Words: gender preselection; DNA analysis; nozzle; sorting

INTRODUCTION

The physical separation of semen into viable X- and Y-chromosome–bearing sperm populations using flow cytometry and sorting has proven to be effective for several species of domestic animals, including rabbits (Johnson et al., 1989), swine (Johnson 1991; Rath et al., 1997), cattle (Cran et al., 1995; Seidel et al., 1997), and sheep (Johnson 1992; Cran et al., 1997; Catt et al., 1997). Human offspring have also been born using this sexing procedure (Fugger et al., 1998. Sorting of viable sperm is performed with a modified flow cytometer/cell sorter (Johnson and Pinkel, 1986), using the DNA content of sperm as the discriminatory parameter. The success of the sorting process is dependent on the accuracy and efficiency of the sperm analysis for DNA. The precision of the DNA analysis is necessarily high (CV < 1.2%) as the difference in DNA content is small (Pinkel et al., 1982; Johnson and Pinkel, 1986; Johnson, 1995). Consistent high resolution measurements can also be obtained by using the slit-scanning technique (Rens et al., 1996), however application for commercial sorting is not feasible.

One of the characteristics of sperm separated on the basis of DNA content using flow cytometric sorting is the relatively low numbers produced per unit of time ($\sim 3 \times 10^5$/hr; Johnson et al., 1989). To overcome the low numbers various alternatives to artificial insemination have been used. Surgical insemination has been used for producing offspring in swine, rabbits and sheep while in vitro embryo production has been used in cattle and swine while intra-cytoplasmic sperm injection (ICSI) has been used for sheep and cattle. Most recently deep uterine insemination was found effective for producing sexed offspring in cattle using ($\sim 2 \times 10^5$ sperm; Seidel et al., 1997). Numbers of sperm used in the various systems vary: surgical insemination (3×10^5 per oviduct; Johnson et al., 1989) in vitro fertilization (2×10^4 per egg; Cran et al., 1995) to 50–250 sperm per egg (Rath, et al., 1997; Long et al., 1998) ICSI using a single sperm (Catt et al., 1997; K Hamano, personal communication regarding cattle ICSI, 1998). The success of fertilization with any method with the exception of ICSI is influenced by the number of sperm available

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and the time interval between the start of the sorting process and the time sperm are delivered to the site of fertilization. The number of sperm available would be increased and the time interval decreased if it were possible to increase the sperm sort rate by increasing the efficiency of sperm analysis/sorting, e.g., increase the fraction of oriented sperm.

Improved efficiency of sperm head orientation is the key factor to increased yields of X and Y sorted sperm. Due to the sperm's flat ovoid shape and compactness of chromatin, only sperm with the proper orientation to the intersecting laser beam during detection can be effectively analyzed for DNA content of sperm heads (Johnson and Pinkel, 1986) and of intact sperm (Johnson et al., 1989). Increasing the proportion of sperm that have their edge properly aligned (oriented) to the 90° fluorescence detector of the sperm sorter is necessary for increased production of sorted sperm. Previously we have used a beveled needle on a commercial sorter (Johnson and Pinkel, 1986) to enhance the yield over random orientation. Characteristic orientation of intact sperm passing through the system is 20% to 80% (Johnson et al., 1989) using the beveled needle. Recently we have improved the proportion of sperm aligned with their edge to the 90° fluorescence detector by two to three times using a newly developed elliptical nozzle (Rens et al., 1998). The new nozzle is capable of moving the forces of orientation much closer to the exit orifice of the nozzle and thus maintaining proper orientation of a greater proportion (60% to 70%) of sperm as they pass the laser beam and thus discarding less sperm due to misorientation.

This paper describes the application of a new elliptical nozzle on a standard speed cell sorter modified for sorting sperm. The nozzle is designed to significantly improve sperm orientation during passage through the sperm sorter. The characteristics and performance of this new nozzle are investigated for sorting viable X- and Y-chromosome–bearing sperm, especially with regard to sperm orientation, DNA measurement and sorting efficiency.

MATERIALS AND METHODS

Sperm Preparation and Staining

Ejaculated semen from 15 mature bulls, three boars, and three rabbits, maintained on regular sperm production schedules, were used for this study. In addition, mouse and human semen samples were analyzed to assess the effect of differing head morphology on the improved nozzle design. Sperm preparation and staining were based on methods described by (Johnson et al., 1987, 1989; Johnson, 1991). Briefly, aliquots of neat semen were extended to a concentration of 15×10^6/ml in Hepes buffered medium containing 0.1% BSA (pH = 7.4) for bull sperm, Beltsville TS extender (BTS, pH = 7.2) for boar sperm, and Tris buffer (0.21 M Tris, 58 mM glucose, and 67 mM citric acid; pH = 6.9) for rabbit sperm (Johnson et al., 1989; Johnson, 1991). Mouse and human semen were also extended in BTS. Sperm were subsequently stained with 7.1 μM Hoechst 33342 (Calbiochem-Behring Corp., La Jolla, CA) and incubated over a 40-min period at 32°C. For the bull sperm studies, just prior to analysis, propidium iodide (1.5 μM, Calbiochem-Behring Corp.) was added to the Hoechst 33342 stained sperm. This allowed dead sperm to be distinguished from living sperm (Johnson et al., 1994). This removal of dead sperm improves sorting purity and efficiency. Stained sperm were also visually examined to determine the percentage of motile sperm using a Zeiss Axiophot fluorescence microscope (365 BP excitation filter and 420 LP filter for Hoechst 33342 fluorescence, 450–490 BP excitation filter and 520 LP filter for PI fluorescence; Carl Zeiss Inc., Thornwood, NY).

Flow Cytometry

Sorting sperm. An EPICS 750 series flow cytometer/cell sorter (Coulter Corp., Miami, FL) was previously modified for flow sorting sperm (Johnson and Pinkel, 1986) and used to separate viable X from Y sperm using the Beltsville Sperm Sexing Technology (Johnson et al., 1989). The modifications were designed to minimize the orientation artifact caused by differential emission of fluorescence from the edge of the sperm compared to the face of the sperm, and therefore to increase the accuracy of measurement of relative sperm DNA content. Briefly, the modifications consisted of the addition of a second fluorescence detector, in the forward position in addition to the standard right angle detector, and a beveled sample injection needle. The alternative system introduced in this paper used a novel nozzle as a replacement for the standard nozzle (Rens et al., 1998) in combination with a standard cylindrical needle rather than a beveled needle to orient sperm. The fluorochrome of the stained sperm was excited with ultraviolet light (UV, 351, 364 nm multi-line) from a 5W 90–5 Innova argon-ion laser (Coherent, Palo Alto, CA) operating at 175 mW. UV blocking filters (418 LP) were used in both fluorescence detectors. Approximately 100,000 sperm containing the X- or Y-chromosome were sorted (Johnson et al., 1989) into separate 0.65 ml micro (presiliconized) centrifuge tubes (single drop sorting). Sorted sperm samples were pulse sonicated to remove sperm tails and restained to approximately the original Hoechst 33342 concentration and reanalyzed on a second flow cytometer (EPICS V, Coulter Corp.) modified for sperm. Resulting DNA histograms were analyzed by fitting them to a pair of Gaussian distributions for purity determination (Johnson et al., 1987, 1989).

New orienting nozzle. The novel nozzle was based on the standard Coulter EPICS V/750 series nozzle (76 μm flow cell tip, catalog no. 6602836), but instead of a tapered circular, shaped interior, the inside of the nozzle had been formed into a tapered elliptical shape (Rens et al., 1998). In this way sperm were oriented deep into the nozzle. The sperm exited the nozzle, through a round 76 μm jeweled orifice into air and passed through the laser beam. The novel nozzle was

used in combination with a standard cylindrical sample injection needle (Coulter Corp.) to orient sperm.

Experimental Design

Several experiments were conducted to investigate the characteristics of the novel nozzle when applied for viable sperm sorting. First the performance of the new nozzle was compared with a standard flow cytometry system and with a modified system using a beveled needle (Experiment 1). Additionally the influence of sperm motility on orientation was analyzed (Experiment 2). Motility is an important feature for viable sperm sorting, as only motile sperm samples (>70%) are considered suitable for sexing and subsequent fertilization. Experiment 3 was designed to assess the influence of sample rate on orientation. Sample rate is important as it is advantageous to sort sperm in the shortest amount of time possible. In experiment four, sperm collected from rabbits, mice and humans were analyzed to investigate the orientation performance of the novel nozzle for these species due to their contrasting sperm morphology.

Experiment 1. Semen of eight different bulls was analyzed on two different days with the standard nozzle and standard cylindrical needle. Semen of 15 different bulls was analyzed on three different days and measured with the standard nozzle and beveled needle, and also with the new nozzle and standard cylindrical needle.

Sample rates were about 2,000 sperm per second. Window settings to select oriented sperm signals were the same for each experiment. Measurements of sperm with the standard and new nozzle were done with different cell sorters (an EPICS V and EPICS 750 series). In this way, low proportions of oriented sperm caused by changing needles back and forth was avoided.

Experiment 2. Measurements were performed with the orienting nozzle. Proportions of oriented intact viable sperm were compared with proportions of oriented sperm without their tails and proportions of oriented dead intact sperm. Measurements were performed on four different days with semen of different bulls. Sample rates were 2,000 sperm per second. Viable sperm could be analyzed separately from dead sperm by their differential Hoechst fluorescence, because PI, which only stains dead sperm partially, quenches Hoechst fluorescence thus forming a separate population of dead sperm (Johnson et al., 1994).

Experiment 3. Semen of eight bulls was measured on two days with sample rates of 500 per sec and 2,000 per sec.

Experiment 4. Semen of three different rabbits was analyzed with the orienting nozzle and proportions of correctly oriented sperm were determined. Additionally, mouse sperm and frozen-thawed human sperm were analyzed to demonstrate the use of this nozzle for these species.

Experiment 5. This experiment was performed to test the purity of the sperm sample sorted with the new nozzle. An improvement in orientation of sperm is only valuable for sperm sexing if (a) The orientation does not decrease the accuracy of DNA measurement and (b) sort stability is maintained. Sorting of X and Y bearing sperm was performed on four different days for bull sperm and three different days for boar sperm. Only samples with 70% or more motile sperm were used. Reanalysis was performed to determine the purity of the sorted sperm populations.

Statistical analysis was performed using Student's *t*-test for small samples (Steel and Torrie, 1960).

RESULTS

Experiment 1

A large improvement in bull sperm orientation was achieved using the novel nozzle compared with the standard nozzle fitted with a cylindrical or beveled needle (paired difference *t*-tests, $P < 0.05$, separate data not shown). On average, a 3.0 times larger proportion of oriented sperm was obtained with the novel nozzle (52.5 ± 4.7%, n = 15) when compared with standard nozzle and standard cylindrical needle (17.3 ± 0.7%, n = 8) and a 2.3 times larger proportion of oriented sperm when compared with the beveled needle (22.7 ± 3.5%, n = 15).

Experiment 2

Proportions of oriented viable bull sperm (56.8 ± 6.7%) were the same as those of tailless bull sperm (59.8 ± 4.2%) or dead bull sperm (53.3 ± 4.3%, no significant difference at $P = 0.05$, n = 4), showing that motility has a negligible influence on orientation of bull sperm when the novel nozzle is used.

Experiment 3

This experiment was performed to investigate the influence of sample rate on orientation. Orientations of 52.1 ± 6.4% and 52.3 ± 6.5% were obtained for sample rates of 500 per sec and 2,000 per sec, respectively (n = 8, paired difference *t*-test, $P = 0.05$, separate data not shown), demonstrating that orientation was not influenced by sample rate.

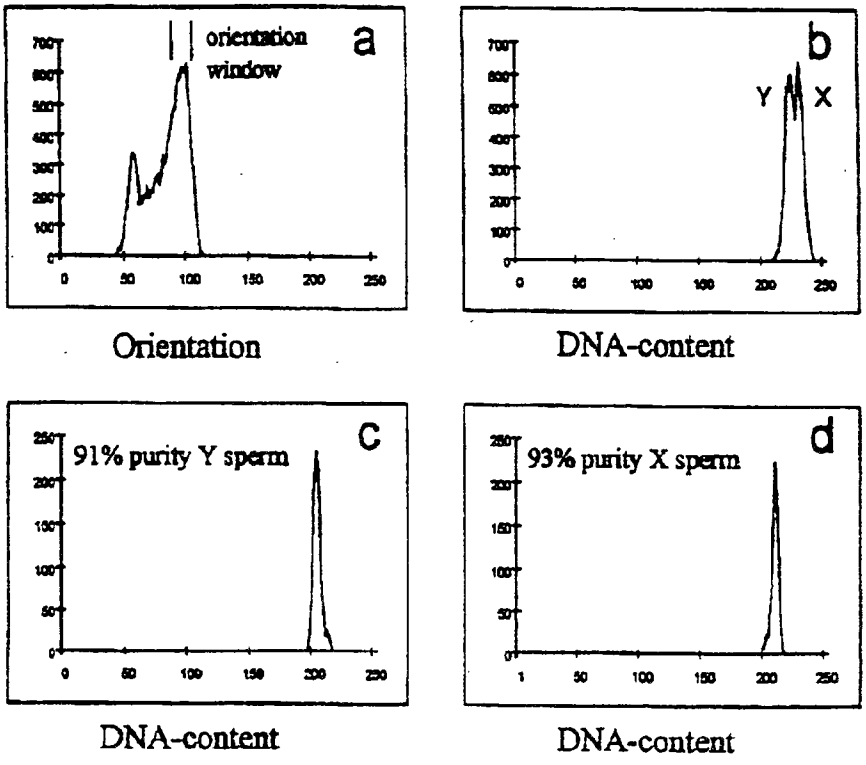
Experiment 4

A high proportion of rabbit sperm were correctly oriented when measured with the novel nozzle (48 ± 2.4% n = 3). The proportion of oriented sperm obtained with mouse sperm and human cryopreserved-thawed sperm was 44% and 45%, respectively.

Experiment 5

Excellent sort stability was maintained with the novel nozzle. Droplet streams as well as droplet delay were stable during sorting with recoveries over 90% using single drop deflection. Bull sperm orientation was on average 54.5% which is about the same as the results of experiments 1–3. High purity sorted samples were obtained on all four days of the experiment (Table 1). The mean for X sperm sort purity was 87.6% and for Y-sperm 89.3%.

Bovine sperm



Porcine sperm

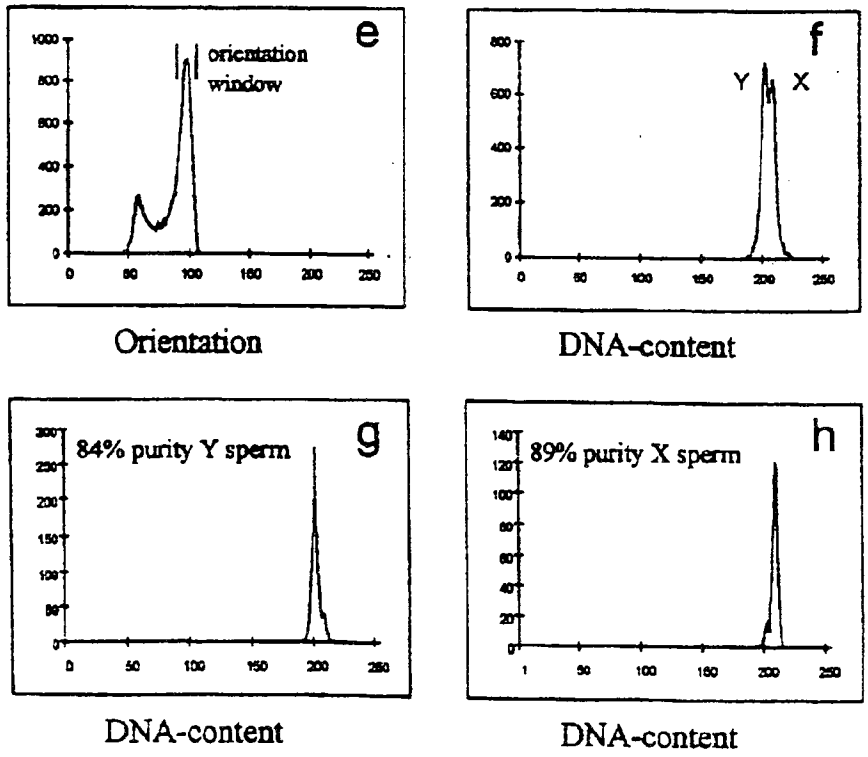


Figure 1.

TABLE 1. Sort Purities of Bovine X-Sperm and Y-Sperm Measured With the Novel Nozzle

Day	Orientation (%)	X-sort (%)	Y-sort (%)
1	50	88.0	90.5
2	57	91.5	90.5
3	59	84.0	85.5
4	52	87.0	90.5
Mean ± sd	54.5 ± 4.2	87.6 ± 3.1	89.3 ± 2.5

TABLE 2. Sort Purities of Porcine X-Sperm and Y-Sperm Measured With the Novel Nozzle

Day	Orientation	X-sort (%)	Y-sort (%)
1	61.5	84.0	89.0
2	60.0	84.3	87.3
3	60.0	87.7	92.0
Mean ± sd	60.5 ± 0.9	85.3 ± 2.1	89.4 ± 2.4

A three-day sorting experiment was also performed with boar sperm. The mean X sperm sort purity was 85.3% and the Y sperm sort purity was of 89.4% (Table 2). Proportions of oriented sperm averaged 60.5 ± 0.9%. Orientation histograms (90° fluorescence) obtained with the novel nozzle and the beveled needle system are presented in Fig. 1 show the significant improvement in orientation that can be achieved with boar sperm with the beveled needle and the new orienting nozzle.

DISCUSSION

The sorting of sperm into X- and Y-chromosome bearing populations with a flow cytometer/cell sorter is based on their differential DNA content. Sperm are stained with the DNA binding fluorochrome, Hoechst 33342 and the fluorescence is collected and measured. However, in order to precisely distinguish X and Y sperm it is necessary to analyze the forward fluorescence of only correctly oriented sperm i.e. sperm with their narrow edge directed toward the 90° fluorescence detector. The reason for this is that the fluorescence is not uniform due the flat ovoid shape of the sperm head and the compactness of its chromatin. Consequently, a brighter fluorescence is exhibited from the edge than flat side. In an unmodified flow cytometer/cell sorter, sperm traverse the laser beam in a random orientation resulting in little or no differential X-Y DNA analysis.

To improve analysis efficiency, the proportion of oriented sperm can be increased by using a beveled sample injection needle (Johnson and Pinkel, 1986; Johnson et al., 1989). The sample core leaving the beveled needle is in the shape of a ribbon, which applies orienting forces to the sperm. However this ribbon only exists when the sample stream is narrow (this means a low sample pressure causing a low sample rate), which is disadvantageous for efficient sperm sorting. Orientation of intact sperm is also more difficult than orientation of sperm heads (tailless sperm) due to the effect of the tail while in fluid motion (Johnson et al., 1989).

To overcome both problems, a novel nozzle was designed to orient sperm. The novel nozzle had the additional advantage that problems associated with placing a beveled needle in the right position were avoided, because the modified EPICS nozzle is screwed onto the flow cell providing the same nozzle position each time it is removed and replaced. The inside of the nozzle was modified such that sperm are subjected to orientation forces from the time they leave the injection needle until the time they enter the jewel and exit the orifice. To guide sperm to the slit, the inside of the nozzle had a specific asymmetric tapered elliptical shape (Rens et al., 1998). Orientating sperm in this way could induce several problems. A wider sample stream or turbulence could affect the accuracy of fluorescence analysis. Although the orifice of the jewel was still round, the slit could have been problematic for sort droplet formation. Therefore, together with studies to investigate improvement of orientation, sort studies were carried out to investigate these concerns, because the main purpose was the efficient sexing of sperm.

In the first series of experiments, orientation measurements with the new nozzle were compared with a conventional system: a standard nozzle and a standard sample injection needle (in a conventional system, sperm traverse the laser beam with random orientation). The difference in outcome between these two systems was a threefold increase in the proportion of oriented sperm. A two to three fold increase in efficiency was found when the new nozzle was compared with the beveled needle system. Experiment 2 showed that sperm motility had no influence on orientation of bovine sperm, with tailless sperm and dead intact sperm having the same proportions of oriented sperm as viable sperm. Thus the use of the novel nozzle should lead to less day to day variation in the percentage of oriented bovine sperm. At flow rates of 2,000 sperm per sec the new nozzle was clearly superior to the beveled needle in percentage of sperm oriented (54% and 23%) respectively. This translated to a sort rate of 200 sperm sorted in each direction for the novel nozzle, double that with the beveled needle.

The influence of sample rate was tested in Experiment 3. A low sample rate (500 sperm per sec) resulted in the same proportion of oriented sperm as a high sample rate (2,000 sperm per sec). Initial experiments with even higher sample rates (3,000–4,000) gave similar results (data not shown). When a beveled needle is used to orient sperm the fraction of oriented sperm decreases with increasing sample rate. This means that the gain in proportion of oriented sperm is higher with higher sample rates which is beneficial for efficient sperm sorting.

The final experiments investigated the effect of the new nozzle on DNA analysis accuracy and sorting behavior. The effective separation of X- and Y-chromosome bearing sperm by sorting is a prerequisite for practical application of the new orienting nozzle. All samples were run with a sample rate of 2,000 per sec. Window settings were the same as in the control sorting

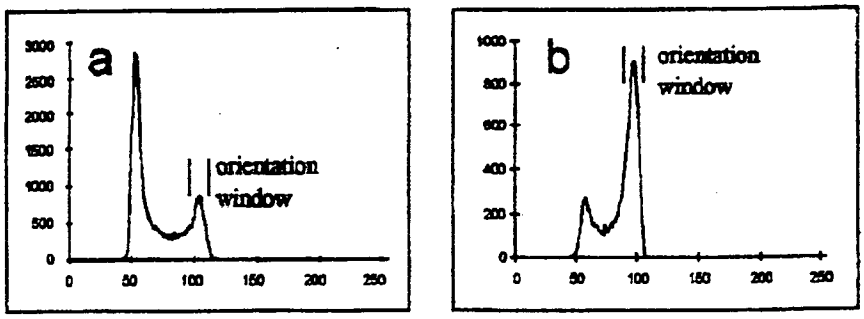


Fig. 2. Orientation histograms of boar intact sperm measured with the beveled needle system (a) or with the novel nozzle (b). The novel nozzle has a superior orientation performance.

procedure (standard nozzle, beveled needle). The proportion of oriented bull sperm for bull was over 50%, and sorting purities were high (about 88%), showing that analysis and sorting accuracy was maintained. The purities obtained were not different ($P = 0.05$) from those previously published (90.3% X-sperm, and 91.6 % Y-sperm, n = 11) using the modified EPICS 750 series flow cytometer/cell sorter with a beveled needle for sperm orientation. Sorting for that study was performed to obtain offspring after deep uterine artificial insemination with X- and Y-bearing bovine sperm (Seidel et al., 1997).

Also boar sperm, which have less difference in X-Y DNA-content than bull sperm (3.6% vs. 3.8%), were sorted into X and Y bearing sperm populations with high purities. The actual rate of sperm deflected and collected as a putative X and Y population (200 per sec, bull sperm; 180 per sec, boar sperm) were on average twice that obtained using only the beveled needle for orientation.

The numbers presented in this paper for proportions of oriented sperm obtained with the new nozzle average about 55%. However, Fig. 2 shows that part of the sperm population are almost oriented, their signals fall just outside the orientation window. The proportion of oriented sperm would be about 75% if this part could also be used for forward (0°) fluorescence analysis. This fact together with the discussed possibility of higher sample rates would lead to a large or increase in efficiency.

Fig. 1. Results of a bovine and porcine X-sperm and Y-sperm sorting experiment using the novel nozzle. a, e: Orientation histograms, only sperm with fluorescence signals within the presented window are used for "DNA" analysis. b, f: DNA histograms, both bovine and porcine histograms show a clear X-Y separation. c, d: Reanalysis histograms of bovine sorted Y sperm (c) and X sperm (d), both are sorted with high purities. g, h: Reanalysis histograms of porcine sorted Y sperm (g) and X sperm (h). They also are sorted with high purities.

The more than two fold increase in yield of sorted X- and Y-chromosome bearing sperm plus the prospect of a further increase will make artificial insemination with sexed sperm more feasible. The gender preselection procedure will be less limited to IVF and embryo transfer or surgical insemination allowing a wider application of sexed semen in the livestock industry. In recent months the new nozzle has been fitted and adapted to a high-speed cell sorting system (MoFlo; Cytomation Inc., Ft. Collins, CO). Preliminary results show that the new nozzle amplifies the sorting capability so as to be capable of even greater increases in sorting efficiency (Johnson et al., 1998).

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Exhibit C

Via Electronic Filing

IN THE UNITED STATES PATENT AND

TRADEMARK OFFICE

Application Number:	09/744,675
Applicants:	Edward L. Squires, Patrick M. McCue, George E. Seidel
Filed:	January 29, 2001
Title:	Equine System for Non-Surgical Artificial Insemination
TC/A.U:	1634
Examiner:	Carla J. Myers
Assignee:	XY, Inc.
Attorney Docket:	XY-Equine3-USNP
Customer No.:	33549

STATEMENT PURSUANT TO

37 C.F.R. § 1.104(c)(4)(iii) AND MPEP § 706.02(l)(2)

U.S. Patent No. 6,149,867, issued November 21, 2000, entitled "Sheath Fluids and Collection Systems for Sex-Specific Cytometer Sorting of Sperm" and the invention claimed in U.S. Patent Application No. 09/744,675, filed January 29, 2001, entitled "Equine System for Non-Surgical Artificial Insemination" were made by or on the behalf of parties to a joint research agreement, within the meaning of 35 U.S.C. 103(c)(3) and 37 C.F.R. 1.104(c)(4)(ii), that was in effect on or before the date the claimed invention was made, and the claimed invention was made as a result of activities undertaken within the scope of the joint research agreement.

Dated this 30th day of July, 2007.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

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